CHANGES IN SOIL MICROBIAL COMMUNITIES DURING SUBSURFACE DECOMPOSITIONS

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Abstract

A cadaver is an energy source with an important role in nutrient recycling. Its decomposition is a complex process attributable to microbial, vertebrate and invertebrate scavenger metabolic activities, which impact the surrounding environmental microbiota with potentially novel forensic applicability if key knowledge gaps are addressed. To investigate subsurface microbiome shifts as a novel tool to establish postmortem microbial clock and augment postmortem interval (PMI) estimations, four studies were made with pigs /piglets as human taphonomic proxies. Study I used a pig leg (*Sus scrofa domesticus*) buried in sandy loamy soil in a sealed polyethylene container for 98 days while Study II compared *S. scrofa domesticus* and plant litter (*Agrostis*/*Festuca* spp) decomposition over 365 days in triplicate microcosms. Study III (300 days) examined two decomposition substrates (whole piglets and *Agrostis*/*Festuca* spp), individually and together. Finally, an *in situ* Study (IV) consisted triplicate burials of piglets and Oak (*Quercus robur*) leaf litter were monitored for 270 days.

For the studies, a combination of environmental variables and molecular analyses were employed. Specifically, analyses/interpretation of the PCR-DGGE fingerprints by richness, Shannon-Wiener and Simpson ecological indices and Illumina Miseq next-generation sequencing were used to determine decomposition-mediated dynamics on soil microbial community diversity, structure and composition. Overall, both pH and temperature changes aligned with microbial community profile shifts and were important variables for PMI estimation. The DGGE-based profiling of the 16S rRNA bacterial gene appeared the most suitable for *S. scrofa domesticus* PMI estimation, while ecological indices of the fungal 18S rRNA gene were better indicators of plant litter decomposition. Some unique taxa, such as Sphingobacteriaceae and Xanthomonadaceae, provided preliminary indicators to differentiate between pig and plant vegetation decomposition as illustrated in Study II. Likewise, taxa such as Sphingobacteriaceae and Alcaligenaceae were identified during the summer season as potential indicators for piglet and piglet + plant litter in Study III whereas

Planctomycetaceae and Sphingobacteriaceae were likely indicators to differentiate between the two carbon substrates. Finally, the *in situ* epinecrotic community profiles in Study IV identified Anaerolineaceae and Methylophilaceae as microbial clock indicators for oak leaf litter and piglet, respectively, for decomposition locations in similar soil types. This research programme is one of the first to compare two decomposition substrates in the subsurface to illustrate potential applicability in PMI estimation.

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Table of contents

List of figures

List of tables

Acronyms

ADD	Accumulated degree days
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FAME	Fatty acid methyl esters
GC	Guanine + cytosine
GNSS	Global navigation satellite system
GPR	Ground-penetrating radar
NGS	Next-generation sequencing
NMDS	Nonmetric multi-dimensional scaling
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
PMI	Postmortem interval
RDP	Ribosomal database project
RMA	Repeat measure analysis of variance
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTK	Real-time kinematic
SCC	Spearman's rank correlation coefficient
SPP	Species
SSCP	Single strand conformation polymorphism
TGGE	Temperature gradient gel electrophoresis
t-RFLP	Terminal restriction length polymorphism
UPGMA	Un-weighted pair group by arithmetic average
VOC	Volatile organic compounds

Chapter 1: Introduction[1]

Microbial ecology or 'microbiome science and technology' has a new found role in forensic and crime scene science. Thus, an inherently multi-disciplinary topic, where soil scientists, soil ecologists, microbiologists, molecular microbial ecologists (microbiome scientists), computer scientists and environmental engineers have exchanged knowledge during the past few decades, has the potential to initiate a second molecular revolution in forensic analyses. This is evidenced by the emergence and adoption of several terms and disciplines that have explicit and/or inferred meanings. The applications of these novel disciplines can depend on the researcher or context specific. It is essential, therefore, to provide the background and provenance for each of these by reflecting on their origins as understood from published literature, what their original intended meanings were and their uniqueness and/or overlaps/interfaces.

According to Coyle (2004), "forensic botany" "*is a marriage of many disciplines* ... (that includes) *plant anatomy, plant growth and behavior, plant reproductive cycles and population dynamics, and plant classification schemes (morphological and genetic) for species identification.*" Bock and Norris (1997) included plant ecology in their historical overview that also reflected the application of the discipline in real criminal investigations. This particular reference justifies, therefore, the study of plant-microbe interactions within forensic contexts. Parallel to molecular analyses in other forensic sub-disciplines, Ferri *et al*. (2009) targeted two DNA regions, psbA-trnH and trnL-trnF, of the chloroplast to create a species identification system, which subsequently differentiated plants at family, genus and species levels. Similarly, Ward *et al*. (2005) developed polymerase chain reaction assays for

[1] A substantive proportion of this work was published as follows:

Ralebitso-Senior T.K. and Olakanye A.O., 2017. General Introduction: Method Applications at the Interface of Microbial Ecology and Forensic Investigation. In: Ralebitso-Senior TK (ed.). *Forensic EcoGenomics: The Application of Microbial Ecology Analyses in Forensic Contexts* (pp 1 – 36). London: Academic Press.

the mitochondrial genome and tested these successfully with 25 different species of grass. More recently, Uitdehaag *et al*. (2016) used trace soil samples for parallel terminal restriction length polymorphism (t-RFLP) bacterial community profiling and palynology in a rape case.

As reflected by many researchers (Vass *et al*., 2008; Dekeusshieter *et al*., 2009; Kasper *et al*., 2012; Donaldson and Lamunt, 2013), "forensic chemistry", the use of chemical science in forensic contexts, is probably one of the more established forensic sub-disciplines for postmortem interval (PMI) estimation. It is underpinned, typically, by the use of spectroscopy, spectrometry, chromatography, magnetic resonance and mass spectroscopy, amongst other techniques, to measure the concentrations of a range of decompositional and metabolic products including, but not limited to, volatile organic compounds, fatty acids, lipids and proteins. The biochemical products that are characteristic of specific decomposition stages are also responsible for attracting certain insect species to the decomposing carrion (Arnaldos *et al*., 2005; LeBlanc and Logan, 2010; Paczkowski *et al*., 2012; Tomberlin and Benbow, 2015). This establishes a direct link to "forensic entomology" where the successional entomological clades that colonize carrion/cadaver and contribute to its rate of decomposition have been used widely in aiding PMI calculations, including in real crime scenarios. The colonization trends are determined further by environmental conditions and associated materials such as temperature, sunlight and clothing (Hobischak *et al*., 2006; Sharanowski *et al*., 2008). New areas of forensic entomology research will most likely include microbe-insect interactions (*e.g.* Schmidtmann and Martin, 1992; Tomberlin *et al*., 2012) both in aboveground and subsurface analyses.

A state-of-the-art use of fungi in criminal investigations in the sub-discipline of "forensic mycology" (or "mycoforensics") was presented in a critical review by Hawksworth and Wiltshire (2011) to reflect the research and case work developed prior to the year of publication. The authors then presented a renewed perspective in Hawksworth and Wiltshire (2015) with additional discourse on the importance of also analysing cadaver gut contents to

aid PMI estimations. The importance of the human mycobiome has since been deliberated within the context of human health and disease (Ghannoum, 2016) and has highlighted how studies of this important clade lag significantly behind those of bacteria. Therefore, efforts are being made by forensic microbiologists with studies of specific fungal species on decomposing cadavers (Sidrim *et al*., 2010), functional groups during specific decomposition stages (*e.g.* ammonia and postputrefaction fungi; Carter and Tibbett, 2003; Tibbett and Carter, 2003), and communities in the surrounding decompositional ecosystem/gravesoil (Tranchida *et al*., 2014; Chimutsa *et al*., 2015). As for any emergent science, justified debates on the applicability and limitations of forensic mycology (*e.g.* Ishii *et al*., 2007; Menezes *et al*., 2008; Bellini *et al*., 2016) are essential to inform robust experimental questions and designs to then establish the discipline as a reliable forensic tool.

Ruffell (2010) expanded the discourse on the importance of and requirement for the appropriate use of terminology in forensic science and proposed "microbioforensics" for "*the study of microbiological (spores, pollen, algae) and macrobiological (skeletal remains) materials*". Potentially synonymous and probably used interchangeably with 'microbioforensics', is "microbial forensics". This sub-discipline was first defined by Budowle (2004) as "*a scientific discipline dedicated to analysing evidence from a bioterrorism act, biocrime, or inadvertent microorganism/toxin release for attribution purposes*". The term was used subsequently in a self-titled book (Budowle et al., 2005) as "*the science that will help bring to justice criminals and terrorists who use biological material to cause harm. … it* [the book] *covers a variety of areas from forensic science, to microbiology, to epidemiology, to bioinformatics, and to legal issues*". The term and discipline have since been applied by numerous researchers largely within the biocrime and bioterrorism contexts although Petrisor *et al.* (2006) provided a scientific discipline-based description as "*the focusing of microbiology, virology, biochemistry and molecular biology for use in environmental forensic investigations*".

The term 'forensic ecogenomics' was defined in literature as "*the application of molecular microbial ecology techniques at the interface of (environmental) forensics, microbiology and archaeology*" to "*expand on initial applications of microbiological analyses of soil*" (Ralebitso-Senior *et al*., 2015). Overall, forensic ecogenomics is the study of microbial community dynamics in different environments for forensic diagnostics and is probably interchangeable with "forensic microbiology" as used by Singh and Crippen (2015) and Carter et al. (2017), for example. Therefore, for this research programme, its intended applications and contexts, include characterisation of:

(i) belowground profiling of the "necrobiome", which was defined by Benbow *et al*. (2013) as "*the community of species (e.g., prokaryotic and eukaryotic) associated with decomposing remains of heterotrophic biomass, including animal carrion and human corpses*"; and

(ii) microbial communities of decomposition-impacted ecosystems including soil and water.

Taphonomy is a word that originates from the Greek words *taphos* and *nomos,* which mean burial and a system of law coined by Efrenov in 1940 (Pokines, 2013). It was defined by Haglund and Sorg (1997) as the study of the various processes associated with cadaver decomposition in relation to postmortem interval, causes and mode of death, location of clandestine graves and identification of the dead. These researchers added that forensic taphonomy is an aspect of forensic anthropology that is used to recreate events that occurred during and after death by gathering and analysing decomposing cadaver data, differentiating between perimortem and postmortem changes in remains, and estimating the postmortem interval (PMI). For example, forensic taphonomy has been used to investigate genocide, war crimes and crimes against humanity (Steadman and Haglund, 2005) and mass burial graves of war casualties (Skinner *et al*., 2003; 2007). However, according to Congram (2013), few

published cases are available where lengthy trials of people involved in crimes can be attributed directly to forensic taphonomic analyses.

According to Carter and Tibbett (2008), taphonomy, a branch of palaeontology, was established to understand how the ecology of a site is affected by the presence of decomposing remains and, in turn, how the site ecology affects the decomposing remains. It is probably this last definition that underpins and justifies the forensic ecogenomic tools used in the current PhD programme where microbial communities of the soil directly beneath and/or around a cadaver or its taphonomic proxy (gravesoil) are characterized using a range of what were largely (molecular) microbial ecology techniques.

1.1 Contextual overview of soil science

Soil can be defined as the epidermis of the earth, which is composed of biological, physical and chemical factors that work together as a complete entity (Whitlow, 2001; Minasny *et al*., 2008; Maier *et al*., 2009). The word 'soil' is fundamental to human existence; this is portrayed by its usage in different languages and by different professionals in various contexts (Lavelle and Spain, 2003; Fitzpatrick, 2008). To engineers, soil is a material that possesses some unique characteristics such as permeability, compressibility and strength to support building structures. To farmers, pedologists and agronomists, soil is a medium where there are intricate interactions between plants, organisms and organic matter (Whitlow, 2001; Schaetzl and Anderson, 2005; Fitzpatrick, 2008). Hydrologists regard soil as a reservoir and medium of water supply (Schaetzl and Anderson, 2005), while forensic science practitioners recognise soil as a vital piece of evidence in crime scene investigations (Petraco *et al*., 2008; Wood *et al*., 2014).

Generally soil texture is indicative of the proportion of sand (0.02 mm – 2 mm), silt (0.002 mm – 0.02 mm) and clay (<0.002 mm) within a soil matrix (Figure 1.1). Soil texture is very important because it helps in understanding the ability of soil to retain nutrients and water. For example clay retains more water than both silt and sand because it has a larger surface

area to volume ratio. Therefore soils with high clay content will retain more water and this subsequently affects both the chemical and physical properties of such soils (Bardgett, 2005; Pepper and Brusseau, 2006). According to Tumer *et al*. (2013), the rate of organic matter mineralisation/decomposition can be affected by soil properties and texture. They assessed the rate by comparing clayey, loamy, organic and sandy soils and observed a high mass loss in both loamy and organic soils.

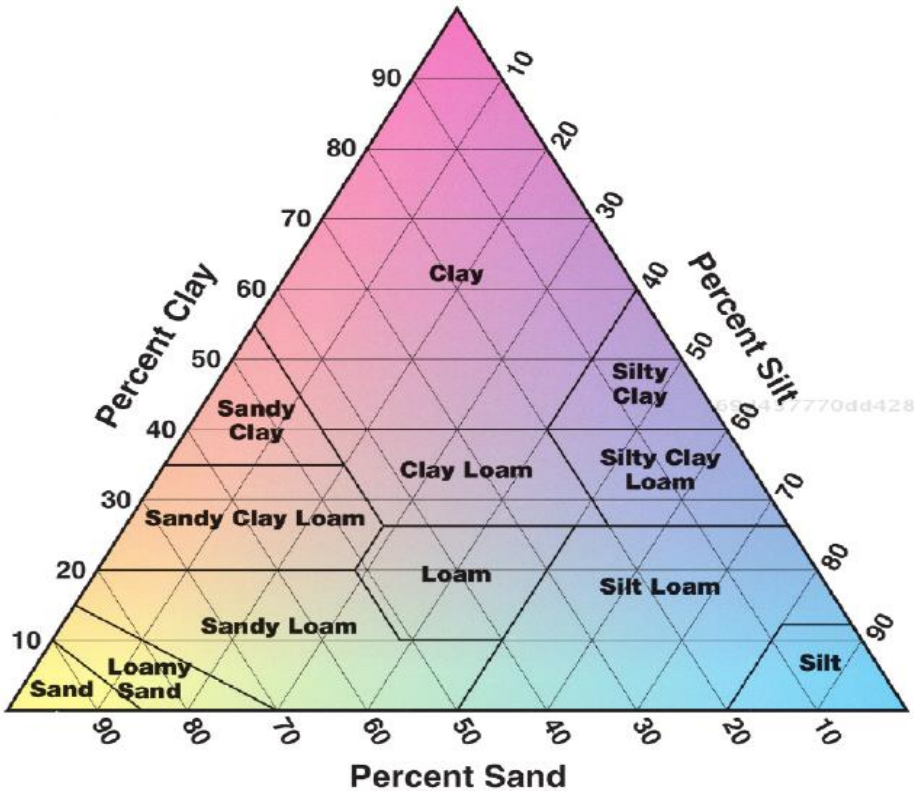


Figure 1.1: Soil texture triangle (Pepper and Brusseau, 2006).

Soil contains organic matter at different stages of decay, and different contents of minerals, water, macro-/microorganisms and gases within its various pore sizes (Schaetzl and Anderson, 2005). Specifically, soil organic matter is composed of two core elements, carbon (C) and nitrogen (N). As reported by various workers (*e.g.* Tibbett *et al*., 2004; Conklin 2005; Hopkins, 2008), the carbon to nitrogen (C:N) ratio is pivotal in understanding soil analysis during decomposition of organic matter as it provides the index for substrate quality. According to Conklin (2005), the C:N ratio of soil organic matter is typically between 10:1 and 12:1. This changes, however, when organic matter with a high C:N ratio is added to soil

where the microbial communities mineralise the available nitrogen rapidly leading to low residual concentrations of soil inorganic nitrogen, N immobilisation within the cells and a slower rate of catabolism/decomposition. In contrast, the addition of low C:N organic matter leads to excess N mineralisation and rapid decomposition (Tibbett *et al*., 2004; Conklin, 2005). Furthermore, Hopkins (2008) stated that the presence of structural proteins in animal biomass results in lower C:N ratios compared to plant biomass.

1.2 Cadaver and cadaver decomposition

According to Daman and Carter (2013), death is the irreversible cessation of the brain and the circulatory and respiratory systems of the body. It is a dynamic process that can occur in seconds or be prolonged (Forbes and Carter, 2015). A cadaver is a dead human body consisting of lipids and proteins, a high water content, microorganisms and a low carbon to nitrogen ratio (Carter and Tibbett, 2008). After death, a cadaver undergoes a series of complex biochemical and pathological breakdown processes ranging from depletion of tissue oxygen by internal microorganisms to autolysis of tissues and cells, and invasion by external microorganisms and scavengers (Carter and Tibbett, 2008; van Belle *et al.*, 2009; Zhou and Byard, 2011). As reported by Janaway (1996), dropping of the lower mandible and loss of eyelid tension are two of the immediate effects that denote death. Subsequent to this, cadaver decomposition, a destructive process, begins approximately after four minutes. This process manifests more microscopically than macroscopically and progresses through various stages of autolysis, putrefaction and decay (Vass *et al*., 2002; Carter and Tibbett, 2008; Forbes and Carter, 2015). Decomposition is a complex process mediated by enzymatic digestion and microbial activities coupled with pertinent environmental conditions, which eventually lead to nutrient migration from the cadaver to the surrounding ecosystem (Vass *et al*., 2002). According to Vass *et al*. (2002), cadaver decomposition is affected more significantly by temperature than moisture. In contrast, Carter *et al*. (2010) stated that the relationship between decomposition and temperature can be modified by soil moisture while soil microorganisms play an important role in cadaver decomposition.

1.3 Stages of cadaver decomposition

As introduced above, cadaver decomposition is a complex phasic process before skeletonisation and takes days, months or years to complete. In the first few hours after death, the body undergoes changes, which are of particular importance in postmortem interval estimation. These changes are often called the "mortis triad" and they entail algor mortis, livor mortis and rigor mortis:

(i) Algor mortis – This is the process of body cooling after death when it loses temperature from the normal average of 37°C until it reaches ambience with the surrounding environment. It is used to estimate time after death but is dependent strongly on the surrounding environmental conditions (Forbes, 2008; Tibbett, 2008).

(ii) Livor mortis – This is also known as lividity and is the settling of blood in capillaries and veins as a result of gravitational pooling (Tibbett, 2008). Livor mortis becomes noticeable between 20 minutes and 10 hours with the formation of purple-red skin discolouration, with maximum lividity occurring between six and twelve hours after death. This has been used effectively by pathologists to estimate death time and establish body repositioning in postmortem analysis.

(iii) Rigor mortis – This stage has been attributed to chemically-mediated muscle and joint stiffening due to a decline in cellular ATP and increase in cellular pH (Forbes and Carter, 2015). The conversion of ATP to ADP increases lactic acid production and results in high cellular pH (Janaway *et al*., 2009; Bristow *et al*., 2011). Rigor mortis becomes noticeable in two to three hours after death by the stiffening of the facial muscles followed by the entire body within 12 hours (Forbes, 2008; Gunn, 2009). After death, the intracellular calcium ion concentration increases due to cell membrane rupture and causes binding of the muscle filaments myosin and actin in the absence of the regulatory proteins tropomyosin and troponin. The result is

stiffening of the dead muscles (Gunn, 2009). The reversibility of rigor mortis is thought to be initiated by muscle cell cathepsin proteolytic enzymes which initiate filament separation (Gill-King, 1997). As for algor mortis, the use of rigor mortis for PMI estimation also depends on the surrounding environmental conditions.

In general, cadaver decomposition is controlled by two catabolic processes, autolysis and putrefaction.

1.3.1 Autolysis

Autolysis is described as the enzymatic breakdown of soft tissues within a cadaver (Janaway *et al*., 2009; Forbes and Carter, 2015). Following death, the heart stops beating and the internal aerobic microorganisms deplete body oxygen. This leads to an increase in blood carbon dioxide and a concomitant decrease in pH and, ultimately, cell poisoning (Vass *et al*., 2002; Alaeddini *et al*., 2010), all of which mark the beginning of autolysis. According to Gunn (2009), the rate of cell death differs among tissue types. For example, the brain dies within three to seven minutes while the skin can still be cultured after 24 hours.

During autolysis, cells and organic compounds, such as lipids, proteins and carbohydrates, are catabolised by internal cellular enzymes principally lipases, proteases and amylases. Cell rupture then results with the release of nutrient-rich fluids (Vass *et al*., 2002; van Belle *et al*., 2009). According to Gill-King (1997), cadaver tissue breakdown occurs in the following sequence:

(i) Stomach, intestines, blood, heart and digestive organs;

(ii) Thoracic cavity and lungs;

(iii) Bladder and kidneys;

(iv) Nervous tissues and brain;

(v) Skeletal muscles; and

(vi) Hair, connective tissues and integument.

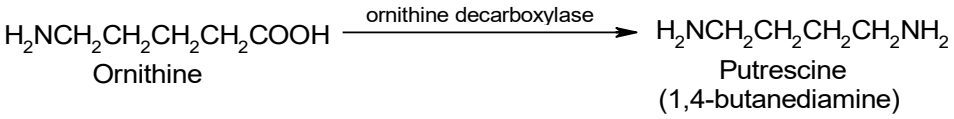
According to Damann and Carter (2013), the intestines, pancreas and stomach are usually the first soft tissue organs to decompose because of the high concentrations of lytic enzymes, while the prostate and uterus are usually the last. Autolysis becomes visible with the formation of fluid-filled swellings on the skin with subsequent sloughing off the body (Vass *et al*., 2002; Forbes, 2008). As reported by Forbes (2008), enteric anaerobic bacteria produce hydrogen sulphide which reacts with deoxygenated blood to blacken the outer blood vessels.

1.3.2 Putrefaction

Putrefaction is soft tissue breakdown into gases, liquids and small molecules by anaerobic microorganisms. It originates primarily from the gut following the generation of anaerobic conditions in the cadaver as initiated by autolysis (Bull *et al*., 2006; Forbes, 2008). The sequential transition from aerobic bacteria, mostly of the phyla Bacteroidetes and Firmicutes, to fermentative anaerobic bacteria, such as *Bacteroides* spp, *Clostridium* spp, *Staphylococcus* spp*, Streptococcus* spp and Enterobacteriaceae, during putrefaction has been reported by many researchers (Janaway *et al*., 2009; Damann and Carter, 2013; Forbes and Carter, 2015). Also reported by Hyde *et al*. (2013) and Pechal *et al*. (2014) are putrefactive bacteria including *Escherichia* spp, *Lactobacillus* spp and *Proteus* spp, which can proliferate on the skin due to the presence of catabolic products of carbohydrates, lipids and proteins (Forbes and Carter, 2015). During the anaerobic state, carbohydrates, lipids and proteins are catabolized to organic acids, such as butyric, lactic and propionic, and gases, including ammonia, hydrogen sulphide, sulphur dioxide and methane.

Some unique features of the putrefactive stage include cadaver swelling, colour change and foul smell (Campobasso *et al*., 2001; Vass *et al*., 2002; Forbes, 2008). An example of the conversion of the amino acid arginine into ornithine by *Enterococcus facealis* enzymes and the further conversion of ornithine into putrescine by *Escherichia coli* was reported by Wilson (2008; cited by Damann and Carter, 2013). The decarboxylation of ornithine and lysine into odorous putrescine (Equation 1.1) and cadaverine (Equation 1.2) was also

reported by (Gill-King, 1997). Consequently, these two products are often detected by cadaver-trained dogs (Gill-King, 1997).



Equation 1.1

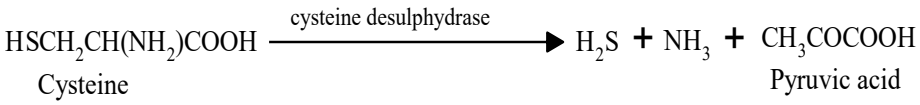
Equation 1.2

An important aspect of putrefaction is cadaver colour change. According to Gill-King (1997), this is due to two decompostional phenomena:

(i) Haemoglobin breakdown and subsequent conversion of haeme to bile pigments; and

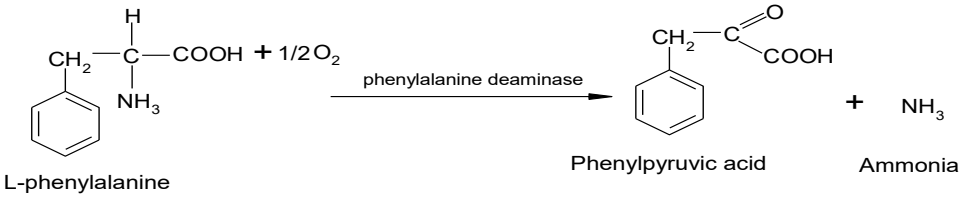
(ii) Formation of sulphide precipitates within tissues and blood vessels.

The first obvious sign of colour change is a greenish-purple skin discolouration resulting from the formation of sulpha-haemoglobin in settled blood. This reaction occurs as sulphur-containing amino acids such as cysteine are catabolised by cysteine desulphydrase to ammonia, hydrogen sulphide and pyruvic acid (Equation 1.3) (Gill-King, 1997).



Equation 1.3

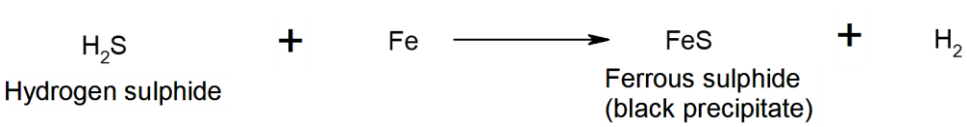
The subsequent reaction of hydrogen sulphide with haemoglobin results in the formation of sulpha-haemoglobin (Clark *et al*., 1997). Also, as reported by Gill-King (1997), the reaction between phenylpyruvic acid and ferric ion in soil causes the formation of a green precipitate on the decomposing cadaver (Equation 1.4).



Equation 1.4

Phenylpyruvic acid + Ferric ion (Fe^{3+}) ⟶ Green complex

According to Damann and Carter (2013), sulphate-reducing bacteria of the order Desulfarcales were identified in grave soils and are known to produce green precipitates that appear early at the right anterior abdominal wall. The breakdown of haemoglobin and other haemoproteins in the liver into different bile pigments also produces colour. For example, biliverdin, a green colour bile pigment, may be reduced to a bilirubin red-colour pigment, which is further reduced to urobilin with a brown colour, while reaction of hydrogen sulphide with iron produces a black precipitate of ferrous sulphide on the cadaver (Equation 1.5) (Gill-King, 1997). According to Damann and Carter (2013), the first appearance of the black precipitate is observed around the ears and nose.



Equation 1.5

As a result of high microbial activity during putrefaction, volatile gases and carbon dioxide are trapped within the cadaver, which causes bloating and swelling (Vass *et al*., 1992). Both propionic acid and butyric acid have been identified during this bloat stage (Vass *et al*., 1992). Also, bloating can be so marked that it results in the displacement of grave earth through abdominal cavity gas expansion (Gill-King, 1997). The subsequent loss of gases then results in soil and body well collapse (Gill-King, 1997; Vass, *et al*., 2002). Prior to this, the presence of gases and fluid accumulation lead to skin rupture and the re-establishment of aerobic conditions (Carter and Tibbett, 2008).

Generally, a temperature range of 21 to 38°C is required for putrefaction to occur, while considerable reductions in the putrefactive process result at temperatures below 10°C and above 40°C (Forbes, 2008).

1.3.3 Decay

The re-establishment of aerobiosis signifies the beginning of decay (Carter and Tibbett, 2008). Proteins and fats decompose to phenolic compounds and glycerol, respectively. Some of the catabolism products identified include putrescine, indole, cadaverine, 3 – methyl indole (skatole) and fatty acids (Vass *et al*., 2002). Overall, decay is a period of rapid

catabolism due to the activities of microorganisms, insects and scavengers (Vass *et al*., 2002; Tibbett, 2008). According to Damann and Carter (2013), the face and head are usually the first points of skeletal exposure although areas of injury or wound may also effect this (Mann *et al.*, 1990).

Although cadaver decomposition is a natural process that starts with autolysis and putrefaction, major breakdown results from the actions of insects and scavengers (Carter and Tibbett, 2008). Many insect species have been identified including blow flies (*Diptera calliphoridae*), flesh flies (*Diptera sarcophagidae*) and beetles (*Coleoptera* spp) (Ururahy-Rodrigues *et al*., 2008; Forbes and Carter, 2015). According to Mann *et al.* (1990), during the decomposition of a cadaver placed outside in a warm or hot environment, it only takes a few seconds for flies to colonise. Fly arrival, coupled with egg laying and the eventual larvae formation, increases the rate of decomposition with the bulk of soft tissue breakdown caused by larvae (Mann *et al*., 1990; Carter and Tibbett, 2008). In a terrestrial environment, scavengers can consume 35 to 75% of the cadaver volume although this may rise during winter when insect and microbial activities slow (Carter and Tibbett, 2008).

The various processes, together with the microbial communities that can be associated with each stage of cadaver decomposition, are summarised in Figure 1.2.

Figure 1.2: An illustration of the stages of cadaver decomposition, microbial communities and metabolites associated with each stage.

1.4 Microbiology of decomposition

Several researchers (Forbes, 2008; Janaway *et al*., 2009; Forbes and Carter, 2015) have suggested that microbial catabolism starts after the cessation of autolysis, which results in a predominantly anaerobic environment that favours gastrointestinal tract and respiratory system bacteria proliferation. According to Janaway *et al.* (2009), "autolysis occurs independently from any bacterial action, while putrefaction, the reduction and liquefaction of tissue, is a microbiologically dominated process." Anaerobic organisms, such as *Bifidobacterium, Streptococcus, Clostridium, Enterobacter* and *Bacteroides species*, enter the tissues and the rest of the body, including bone, via the lymphatic and vascular systems (Gill-King, 1997; Forbes, 2008; Forbes and Carter, 2015). With anaerobic microorganism proliferation, the only viable obligate aerobic microorganisms found on decomposing tissues are *Micrococcus*, *Enterococcus, Pseudomonas* and *Acinetobacter* species (Gill-King, 1997; Janaway *et al*., 2009). Approximately 90% of the culturable microorganisms isolated from decomposing tissues are strict anaerobes, with Gram-positive non-sporulating strains (such

as *Bifidobacterium* spp) giving the highest counts (Janaway *et al*., 2009). Enterobacteriaceae, including *Lactobacillus* and *Streptococcus* spp, have also been observed but in lower numbers. Metcalf *et al*. (2013) and Pechal *et al*. (2014) reported shifts from aerobic bacteria of the Actinobacteria and Firmicutes phyla to dominance of facultative anaerobic bacteria including *Lactobacillus*, *Streptococcus* and *Staphylococcus* spp in the mouth and skin of a carcass. Nonetheless, other bacteria, such as *Bacillus* spp and *Pseudomonas* spp, have been reported (Janaway *et al*., 2009) but in lower numbers. Other documented organisms include: *Proteus* spp*; Salmonella* spp*; Klebsiella* spp*; Serratia* spp*;* and *Flavobacterium* spp (Janaway *et al*., 2009). As reported by Sidrim *et al*. (2010), fungi, such as *Aspergillus* spp, *Penicillium* spp and *Candida* spp, were isolated and identified from human cadavers during the bloat and putrefaction stages. Likewise, Carter and Tibbett (2008) reported several strains of the fungal taxa Ascomycetes and Basidiomycetes in gravesoil.

1.5 Chemistry of decomposition

Cadaver decomposition is a complex process which begins with the initial postmortem mortis triad and is followed by the soft tissue decomposition processes, which are characterised by the catabolism of proteins, carbohydrates and lipids [1.2].

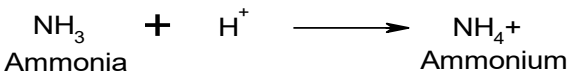
1.5.1 Protein decomposition

Proteins undergo enzymatic degradation at a non-uniform rate by proteolysis (Sabucedo and Furton, 2003; Forbes, 2008). The rate of cadaver proteolysis is dependent on moisture content, temperature and microbial activity (Vass *et al*., 2002). Specifically, the rate is reduced by cooling but enhanced by warming (Janaway *et al*., 2009). The epithelial cells and neurones are usually the first soft tissue proteins to putrefy (Dent *et al*., 2004) although brain, kidney and liver decomposition also occur (Gill-King, 1997). In contrast, proteins such as the epidermis reticulum and muscles are more resistant to proteolysis while connective tissues and cartilage are the most resistant (Gill-King, 1997; Forbes, 2008). Collagen and

keratin in particular exhibit resilience to biological and chemical agents and, consequently, remain intact amongst skeletal remains (Forbes, 2008; Janaway *et al*., 2009). Proteolytic bacteria include *Bacillus*, *Micrococcus* and *Pseudomonas* species (Janaway *et al*., 2009). Together with the above species, gastrointestinal tract sulphate-reducing bacteria also play important roles.

Key metabolites of proteolysis include amino acids, peptones, proteoses and polypeptides. Other metabolites are phenolics and gases such as hydrogen sulphide, carbon dioxide, methane and ammonia (Forbes, 2008). The sulphur-containing amino acids are catabolised by desulphydralation to yield ammonia, hydrogen sulphide, sulphides, pyruvic acid and thiols (Gill-King, 1997; Knight and Presnell, 2005).

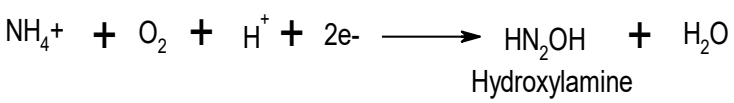
According to Forbes (2008), the sulphide metabolites do not metabolise although they are oxidised to sulphates under aerobic conditions by bacteria such as *Thiobacillus* spp to produce sulphurous acid in soil. Subsequently, the amine components of the amino acids are liberated as ammonia, which then may be converted to ammonium (Equation 1.6) at low soil pH (Gill-King, 1997; Forbes, 2008).



Equation 1.6

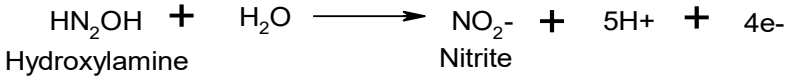
Ammonium compounds promote plant growth, which has consequently become a noticeable feature around or above a decomposing cadaver (Gill-King, 1997; Carter and Tibbett, 2008; Forbes, 2008). According to Forbes (2008), unutilizied ammonium ions can undergo either nitrification or denitrification in soil. Nitrification is the oxidation of the ammonium ion via nitrite to nitrate and this process can be divided into two steps (Equations 1.7 - 1.9) (Killham, 1994);

(i) Ammonium ion oxidation to nitrite by Gram-negative *Nitrosomonas* spp.



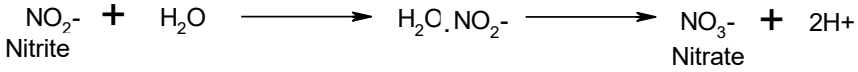
Equation 1.7

Equation 1.8



(ii) Nitrite oxidation by Gram-negative *Nitrobacter* spp

Equation 1.9



These bacteria are sensitive to changes in environmental pH with *Nitrosomonas* spp proliferating between pH 7 and 9 while *Nitrobacter* spp are favoured at pH values between 5 and 8 (Forbes, 2008).

1.5.2 Carbohydrate decomposition

Cadaver carbohydrates such as glycogen are catabolised microbially to glucose monomers, which, in turn, can be mineralised to water and carbon dioxide or oxidised to various alcohols and organic acids (Forbes, 2008). For example, the aerobic catabolism of sucrose by fungi produces citric, oxalic and glucuronic acids while the anaerobic *Clostridium* spp catabolise carbohydrates to alcohols and organic acids, such as acetic, butyric and lactic. The acidic conditions in proximity to a decomposing cadaver have been attributed to these by-products (Forbes, 2008; Janaway *et al*., 2009). Additional bacterial catabolic products include: gases, such as hydrogen, hydrogen sulphide and methane; alcohols, such as ethanol and butanol; and acetone (Forbes, 2008; Janaway *et al*., 2009). According to reports by Collison (2005) and Ziavrou *et al*. (2005), determination of antemortem blood alcohol concentrations remains a problem for pathologists and toxicologists due to postmortem bacterial production of ethanol.

1.5.3 Lipid decomposition

Human adipose tissue is composed of 60 to 80% lipids, with water accounting for the balance. Lipids, in turn, are comprised of approximately 90 to 99% triglycerides (triacylglycerol), which are unchained fatty acid esters of glycerol. Monounsaturated $C_{18:1}$

oleic acid is the most abundant fatty acid in adipose tissue, followed by polyunsaturated $C_{18:2}$ linoleic acid and monounsaturated $C_{16:1}$ palmitoleic acid. The corresponding saturated $C_{16:0}$ palmitic acid is also present (Forbes, 2008). Rothschild *et al*. (1996) reported that the fat content of a cadaver was hydrolysed by intrinsic lipases to fatty acid metabolites, which, in turn, were either oxidised or hydrolysed under aerobic or anaerobic conditions, respectively.

In the presence of oxygen, unsaturated fatty acids are converted to ketones and aldehydes by bacteria and fungi (Forbes, 2008). In contrast, both saturated and unsaturated fatty acids undergo bacterial hydrolysis and hydrogenation under anaerobic conditions (Forbes, 2008). For example, Clostridia produce lipolytic enzymes that facilitate anaerobic hydrogenation and hydrolysis. *Clostridium perfringens,* a major agent of cadaver decomposition, has been isolated from the gastrointestinal tract for its strong proteolytic, lipolytic and saccharolytic abilities (Forbes, 2008; Janaway *et al*., 2009). This bacterium is also found in soils exemplifying its involvement in grave cadaver decomposition (Forbes, 2008). The reduction of cadaver fatty tissues to fatty acids depends greatly on moisture content, more particularly water activity, and catabolic enzyme activity (Forbes, 2008; Janaway *et al*., 2009).

1.6 Adipocere

Various workers have described adipocere as "grave wax" or "corpse wax" (Forbes, 2008; Ubelaker and Zarenko, 2011; Schoenen and Schoenen, 2013) with its definition based on morphological characteristics. Thus, Gill-King (1997) defined it as a "greyish white caseous material", Janaway *et al*. (2009) defined it as a "waxy substance" while Schoenen and Schoenen (2013) described it as a "whitish, soft-smeary up to firm-crumbly, inodorous, mouldy or putrid smelling, fatty feeling lipid bulk". Independent of definition, the basis of adipocere formation, as largely agreed by various researchers, is the hydrolysis and hydrogenation of adipose tissue (Forbes *et al*., 2005a; Ubelaker and Zarenko, 2011; Schoenen and Schoenen, 2013). Bacterial activities in a temperature range of 22 to 38°C, in a damp and anaerobic environment, have been reported to enhance its formation (Forbes,

2008; Schoenen and Schoenen, 2013; Kasuda *et al*., 2016). As reported by Ubelaker and Zarenko (2011), adipocere is an essential taphonomic phenomenon due to its:

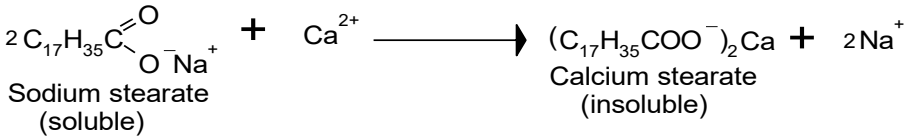
(i) Preservative characteristics of soft tissues;

(ii) Ability to reveal constitutional and environmental factors that may prove valuable in forensic investigation; and

(iii) Complicating role in the determination of postmortem interval due to its preservative characteristics.

Adipocere formation follows triglyceride hydrolysis to free fatty acids with the unsaturated fatty acids converted to saturated fatty acids (Gill-King, 1997). For example, oleic acid hydrolysis and oxidation result in a palmitic acid concentration increase, while hydrogenation of its double bond results in the formation of 10 – hydroxy stearic acid, which is subsequently dehydrogenated to oxo-stearic acid (Forbes, 2008).

The major components of adipocere, as described by Forbes *et al*. (2005a; 2005b) and Ueland *et al*. (2014), are a mixture of saturated fatty acids (stearic, palmitic and myristic), unsaturated fatty acids (linoleic, palmitoleic and oleic) and their calcium salts. According to Gill-King (1997), free fatty acids can attach to interstitial fluid potassium and sodium ions at a "slightly alkaline pH" while the identification of fatty acid calcium salts as adipocere components from grave site samples was attributed to potassium and sodium ion displacement (Equation 1.10).



Equation 1.10

In summary, adipocere formation occurs where there are cadaver fatty tissues or even a minimal fat content such as in internal organs such as the heart, liver and kidneys (Forbes *et al*., 2005a; Forbes *et al*., 2005b; Kasuda *et al*., 2016). As reported by Forbes (2008), *Clostridium perfringens* growth and production of lecithinase stimulate adipocere formation

in anoxic environments. Ueland *et al*. (2014) reported a dominance of Gram-negative bacteria at the later stages of adipocere formation and theorised that this group outcompeted the Gram-positive bacteria over time. With the exception of hot, dry soils, adipocere can be formed in a range of ecosystems. Subsequent adipocere decomposition, as reported by Pfeiffer *et al*. (1998), may be attributed to Gram-positive bacteria such as *Bacillus*, *Cellulomonas* and *Nocardia* species.

1.7 Factors affecting decomposition

Although several factors, including temperature, humidity/water activity/moisture, soil pH, redox potential, trauma, associated material such as clothing and body mass, have been reported to affect cadaver decomposition rate, they vary between aboveground and underground situations (Mann *et al*., 1990; Carter and Tibbett, 2008; Carter *et al*., 2010; Zhou and Byard, 2011). Hence, as reported by workers such as Mann *et al*. (1990) and Carter and Tibbett (2008), cadaver decomposition rate often slows in underground environments. Notwithstanding this, the individual parameters are considered as follows:

(i) Temperature – Ambient temperature increase has the largest effect on body decomposition rate as reported by Mann *et al*. (1990) and Gill-King (1997). According to Carter and Tibbett (2008), an increase in biological activity during decomposition was due to temperature increase. Zhou and Byard (2011) reported that microorganisms use the body as a growth medium after death and the most favourable temperature for putrefactive bacteria (anaerobic catabolic bacteria) occurs between 21 and 38°C while their activities are inhibited at temperatures below 4°C. As observed by both Micozzi (1986) and Stokes *et al*. (2009), the rate of aerobic decomposition is enhanced in a frozen cadaver compared with a fresh cadaver where putrefaction occurs due to anaerobic activity. In an undergound environment, as observed by Carter and Tibbett (2006), microbial acitivity rates

increase as the temperature rises up to 38°C, while at -5°C both enzymatic and microbial activity stop.

(ii) Water activity – A cadaver consists of 60 – 80% (w/w) water, which, according to Carter *et al*. (2010), affects soil microbial activity. Cadaver breakdown is usually described in terms of decomposition and desiccation, where rapid desiccation can inhibit decomposition and lead to mummification (Carter and Tibbett, 2008). Extreme dry conditions dehydrate cadavers rapidly, which results in preservation, whereas extreme wet conditions promote waterlogging and adipocere formation. Both situations slow the decomposition rate (Campobasso *et al*., 2001; Carter and Tibbett, 2008; Zhou and Byard, 2011).

(iii) Trauma – Open wounds or cuts in a cadaver have been reported to promote its decomposition rate (Mann *et al*., 1990; Campobasso *et al*., 2001) since they serve as entry points for external microorganisms and insects.

(iv) Associated material – The presence of clothing on a cadaver has been reported to aid the rate of decomposition. For example, maggots tend to avoid sunlight by gathering under clothing (Campobasso *et al*., 2001; Zhou and Byard, 2011).

1.8 Plant litter decomposition

The decomposition of non-living matter, which was described by Swift *et al*. (1979) as detritus, is an important component of energy and nutrient recycling in ecosystems (Moore and Schindler, 2008; Jia *et al*., 2016). By far, the decomposition of plant detritus (*e.g.* leaves, grasses or dead wood) has been one of the most studied (Swift *et al*., 1979; Gessner *et al*., 2010; Jabiol *et al*., 2014). Typically, the occurrences, distributions, phylotypes and genotypes of the microbial communities involved in these decompositions depend on several factors including: individual plant species or a mixture of different species; soil types; season; plant quality and composition; timeline of leaf shedding; habitat; plant infestation

and disease; climatic conditions during decomposition; and biogeochemical characteristics of the ecosystem (Kominoski and Pringle 2009; Chapman *et al*., 2013; Zhang *et al*., 2015; He *et al*., 2016).

According to Jia *et al*. (2016), the rate of plant litter decomposition is faster in its natural habitat because of the close relationship between the plant species and the degradative soil microbial communities, which was described by Ayres *et al*. (2009) as microbial adaptive mechanism. Leaf litter decomposition as described by both He *et al*. (2016) and Purahong *et al*. (2016), can be divided into two phases. The first phase is regulated by nutrient concentration and carbon availability, which, according to Hyde *et al*. (2017), is dominated by bacterial biomass, while the second phase is regulated by the activities of fungi on lignin decomposition. Decomposition of leaf litter has often been attributed to digestive enzymatic activities of fungi (primary decomposer) that catalyse macromolecules such as cellulose, hemicellulose and lignin (Voriskova and Baldrian 2013; Purahong *et al*. 2016). Across-kingdom activity between bacterial and fungal communities was reported during plant litter decomposition (Purahong *et al*. 2016; Gui *et al*., 2017). Likewise, seasonal microbial decomposer occurrence was reported by Mora-Gomez *et al*. (2016) with bacterial communities associated with summer and fungal communities aligned with spring. Some bacterial taxa associated with plant litter decomposition include Clostridia, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Bacteroidetes and Actinobacteria (Schellenberger *et al*., 2010; Stursova *et al*., 2012; DeAngelis *et al*., 2013) while the associated fungi includes Ascomycota, Basidiomycota and Glomeromycota phyla (Osono 2011, Voriskova and Baldrian 2013).

Although forensic botany addresses surface plant growth as a tool to identify locations of potential criminal burials, very little research exists on the role of subsurface plant litter occurrence and subsequent degradation parallel to human cadaver decomposition. It is highly likely that plant material may be incorporated unintentionally in and/or used

deliberately to mask clandestine burials in crime scenes. Therefore, to address this knowledge gap, comparisons between soil microbial communities associated with subsurface cadaver and litter decomposition processes were made in this research programme to mimic two crime scene scenarios where grass from a domestic garden and oak leaves from a forest were incorporated in clandestine graves.

1.9 Microbial ecology in the forensic context

Earth burial to honour the dead has been practised for thousands of years. Forensically, soil is viewed as important crime scene evidence (Bull *et al*., 2006; Petraco *et al*., 2008; Woods *et al*., 2014) although accurate PMI estimation can be very challenging. Nevertheless, much research has been made to gain a better understanding of this. For example, forensic entomologists have used successfully insect life cycles in minimum PMI estimations (Rodriguez and Bass 1983; Dadour and Harvey, 2008; Al-Mesbah *et al*., 2012). Similarly, Vass *et al*. (1992; 2002) reported the use of cadaver decomposition volatile organic compounds (VOC) from a grave soil in PMI estimation. Furthermore, Benninger *et al*. (2008), studied the biochemical changes below a cadaver by recording shifts in pH and nitrogen and phosphorus contents of a gravesoil at different postmortem time intervals. Indeed, the analysis of multiple bio- and/or physicochemical parameters, including CO_2, NH_4^+, total C, total N, P, K, moisture content, pH and temperature was central to most seminal pre-2010 investigations (*e.g.* Hopkins *et al*., 2000; Tibbett *et al*., 2004; Carter and Tibbett, 2006; Benninger *et al*. 2008).

Billions of microorganisms inhabit terrestrial ecosystems with bacteria the most predominant (Maier and Pepper, 2009). As reported by McGuire and Treseder (2010), 90% of organic matter catabolism can be attributed to bacterial and fungal activities coupled with their important roles in the carbon and nitrogen cycles. Numerically, less than 1% of microorganisms have been characterised by culture-based techiques (Maier and Pepper, 2009; Madigan *et al*., 2012a) so the use of culture-independent tools to characterise

microbial communities structures, compositions and diversities has proven to be very successful (Elsas and Rutgers, 2006). The most frequently used techniques for microbial community characterisation are based on macro cell components such as whole-cell fatty acids and nucleic acids that can be extracted directly from soil.

The use of both culture-based and culture-independent tools to study the relationships/interactions between soil microbiomes and cadaver necrobiomes has identified their potentials to estimate PMI in a forensic context. This was exemplified in a seminal study by Parkinson *et al*. (2009) where two human cadavers were placed aboveground with T-RFLP and phospholipid fatty acid analysis (PLFA) used to profile the bacterial and fungal communities of the soil immediately (0-5 cm) below. The investigation showed changes in both bacterial and fungal communities with successional patterns recorded along the decomposition timeline. As a result, the authors proposed the use of both bacterial and fungal communities in forensic investigation. Post-2010, the application of high-thoughput microbial ecology tools by investigators such as Metcalf *et al.* (2013), who observed microbial community changes in a mouse cadaver model, have identified the potential of the microbial community as a "postmortem microbial clock". Overall, the state-of-the art of microbial ecology analyses within the forensic context, to enhance PMI/postburial interval (PBI) determinations, is summarised in Table 1.1 where the paucity of subsurface decomposition research is also highlighted.

1.9.1 Whole cell fatty acid community profiling

Microbial cell membranes contain lipids where they also act as storage products. Different cytoplasmic lipids that include free fatty acids, fatty alcohols, glycolipids and phospholipids have been identified and used as signature molecules to characterise microbial community structures (Dunfield, 2007; Kandeler, 2007; Madigan *et al*., 2012a). This application can be based on two analyses: phospholipid fatty acids; and fatty acid methyl esters (FAME). The former are good signature molecules since they are found in the cytoplasmic membranes of

viable microorganisms but not as storage products. Although the PLFA extraction from soil samples is a lengthy process, it has been used to characterise different microbial environmental adaptation strategies in a range of soil types (Dunfield, 2007; Kandeler, 2007; Marschner, 2007). According to Kandeler (2007), Gram-positive bacteria produce odd number and branched-chain fatty acids, whereas Gram-negative bacteria produce even number, straight-chain and cyclopropane fatty acids. While there are up to 15 bacterial fatty acid signatures, few are found to be characteristic of fungi, which may lead to an under estimation of the fungal biomass (Marschner, 2007).

Table 1.1: A temporal overview (2006 – 2017) of the application of bio/physicochemical, culture-based and molecular microbial ecology analyses in body/mammalian analogue decomposition studies in a forensic context. N/A denotes no microbial ecology technique(s) applied.

Microbial ecology technique	Study location	Study context	Animal model	Biochemical or physicochemical analysis	Key study findings	Reference
N/A	Underground	Microbial activity	Sheep (*Ovis aries*)	Temperature, CO_2, soil pH, C_{mis}, C & N content	Temperature had effect on rate of tissue mass loss. Correlation between CO_2 evolution and tissue mass loss.	Carter and Tibbett (2006)
T-RFLP & PLFA	*In situ* aboveground	Soil bacteria and fungi	Human cadaver	Not determined	Successional changes in the bacterial and fungal communities.	Parkison *et al.* (2009)
N/A	Surface microcosm	Soil chemistry, microbial activity	Pig (*Sus scrofa domesticus*)	Tissue mass loss, CO_2, soil pH, nitrate, NH_4^+, phosphate, potassium, electroconductivity	Freezing has no effect on decomposition, changes in microbial activity and soil chemistry due to the presence of skeletal muscle tissue.	Stokes *et al.* (2009)
N/A	Above and belowground	Ninhydrin reactive nitrogen (NRN)	Pig (*Sus scrofa domesticus*)	Not determined	Soil NRN analysis can be used within two months for below ground and 97 days for aboveground	van Belle *et al.* (2009)
Culture-based/qPCR	*In situ* aboveground	Soil lipolytic and proteolytic bacteria	Pig (*Sus scrofa domesticus)*	Temperature	Increase in lipolytic bacteria during active decomposition. Decrease in proteolytic bacteria from fresh to advanced decay.	Howard *et al.* (2010)
LH-PCR	*In situ* aboveground	Soil bacteria	Human cadaver	Not determined	Recovered amplicons for anaerobic and nitrogen-fixing bacteria.	Moreno *et al.* (2011)
454 Pyrosequencing (NGS)	*In situ* aboveground	Cadaver bacteria community	Human cadaver	Not determined	Shift from aerobic to anaerobic bacteria between the initial and end point of bloat stage.	Hyde *et al.* (2013)

Illumina (NGS)	Surface microcosm	Soil and carcass epinecrotic community	Mouse	Soil pH	Use of bacteria and eukaryotes as a microbial clock in PMI.	Metcalf *et al.* (2013)
Illumina (NGS)	Surface microcosm	Soil bacteria, archaea and fungi	Mice *(Mus musculus)*	Soil pH	Changes in *Mus musculus* necrobiome during active and advanced decay. Shifts differed between sterile and non-sterile soils.	Lauber *et al.* (2014)
454 Pyrosequencing (NGS)	*In situ* aboveground	Epinecrotic bacterial community	Pig (*Sus scrofa domesticus*)	Microbial metabolic community profiling with Biolog EcoPlates™	Bacterial communities successions at phylum and family taxonomic levels suggested that the shifts could be used to estimate and define unique minimal decomposition intervals.	Pechal *et al.* (2014)
DNA-/RNA-based DGGE	Subsurface microcosm	Soil bacterial and fungal microbiome	Pig (*Sus scrofa domesticus*)	Temperature, soil pH	Microbial diversity changed temporally with decomposition.	Bergmann *et al.* (2014); Olakanye *et al.* (2014); Chimutsa *et al.* (2015)
			Pig (*Sus scrofa domesticus*) / Plant litter (*Agrostis/Festuca* spp)	Temperature, soil pH	Dominance of 16S rRNA gene for *Sus scrofa domesticus* soils. Dominance of 18S rRNA gene for the *Agrostis/Festuca* spp soils.	Olakanye *et al.* (2015)
454 Pyrosequencing (NGS)	*In situ* aquatic environment	Epinecrotic bacterial community	Pig (*Sus scrofa domesticus*)	pH, dissolved oxygen, temperature, salinity, oxidation-reduction potential	Successional changes in the bacteria between summer and winter in the fresh water habitat.	Benbow *et al.* (2015)
Illumina (NGS)	*In situ* belowground	Soil bacterial microbiome	Human cadaver		Dominances of Acidobacteria, where Proteobacteria were identified in microbial succession.	Thomas *et al.* (2017)

Illumina (NGS)	*In situ*	Soil bacterial and fungal microbiome	Pig (*Sus scrofa domesticus)* / Leaf litter (*Quercus robur*)	Temperature, soil pH	Methylophilaceae and Anaerolineaceae identified as potential microbial clocks for pig and leaf litter, respectively.	Olakanye *et al.* (2017)
FAME	*In situ* aboveground	Soil microbial community	Pig (*Sus scrofa domesticus*)	Soil pH, soil moisture	Shift in the FAME profiles aligned with the start of the active decay into the dry period.	Breton *et al.* (2016)
Illumina (NGS)	*In situ* aboveground	Soil microbial community	Pig (*Sus scrofa domesticus*)	Not determined	Seasonal changes associated with postmortem microbial communities. Soil microbes are part of the postmortem microbial community.	Carter *et al.* (2015)
Culture-based analysis	*In situ* aboveground	Culturable aerobic bacteria	Pig (*Sus scrofa domesticus*)	Soil temperature, soil pH and oxidation-reduction potential	Different locations on the taphonomic proxy tended to host different bacteria. The culturable aerobic bacterial communities changed over time.	Chun *et al.* (2015)
N/A	*In situ* aboveground	Soil biochemical analysis	Pig (*Sus scrofa domesticus*)	Soil pH, moisture content, TC, TN, PO_4, lipid-phosphorus	Increase in soil pH and concentration of TN, PO_4 and lipid-phosphorus.	Benninger *et al.* (2008)
Illumina (NGS)	*In situ* aboveground	Soil bacterial microbiome	Human cadaver	Soil pH, TOC, TN, PO_4, NO_3, NH_3	Increases in TOC, PO_4 and NH_3; Changes in functional community composition.	Cobaugh *et al.* (2015)
DNA based	*In situ* aboveground	Soil biochemical analysis and DNA extract	N/A	Moisture content, pH, TOC, TC, TN, lipid-phosphorus in soil	Increases in TN, lipid-P, water and pH when conpared to the control site.	Damann *et al.* (2012)
454 Pyrosequencing (NGS)	*In situ* aboveground	Necrobiome	Human cadaver	Temperature	Bacteria members, *Ignatzschineria* and *Wohlfahrtimonas* spp, associated with flies recorded at bloat stage. Actinobacter recorded	Hyde *et al.* (2015)

					after dehydration and skeletonization.	
Illumina (NGS)	Above and belowground	Soil bacteria	Human cadaver	Not determined	Decreases in diversity, taxa richness and evenness in surface decomposition. Increased taxa richness but decrased evenness in subsurface decomposition. Proteobacteria dominant in both above- and below-ground contexts.	Finley *et al*. (2016)
Illumina (NGS)	Sterile cardboard boxes; Buccal cavity and rectum	Premortem microbiome; Necrobiome	Adult female Sprague Dawley rat carcasses	Not determined	Bacterial communities from the two different body sites became more similar with decomposition. Proteobacteria became more predominant temporally. Sarcocaphagous insects increased mass loss but had no detectable impacts on microbiome profiles.	Guo *et al*. (2016)
Illumina (NGS)	*In situ* aboveground and microcosm	Soil epinecrotic community	Mouse and mammalian corpse	pH, total nitrogen and ammonium concentrations	Seasonal microbial changes with soil as primary sources of decomposers. Soil type had little or no effect on epinecrotic community.	Metcalf *et al*. (2016)
Illumina (NGS)	*In situ* aboveground	Soil epinecrotic community	Pig (*Sus scrofa domesticus*)	Temperature	Difference between the soil epinecrotic community of the grave soil and the control site with the carcass mass having no influence.	Weiss *et al*. (2016)
N/A	*In situ* belowground	Soil biochemical analysis, microbial biomass and activity	Pig (*Sus scrofa domesticus*)	TC, TN, pH, moisture content, NH_4^+, amino acids, microbial biomass and activity	Increases in TC, TN, microbial biomass, activity, amino acids and NH_4^+ of the gravesoil when compared to the control site.	Hopkins *et al.* (2000)

Colony PCR	*In situ* aquatic environment	Marine bacterial	Pig (*Sus scrofa domesticus*)	Not determined	Involvement of saprophytic bacteria in marine decomposition. Bacteria successional and seasonal differences recorded during the study.	Dickson *et al.* (2011)
N/A	*In situ* aboveground	Soil biochemical analysis	Human cadaver	DOC, DON, pH, EC, SO_4^{2-}, NH_4^+, NO_3^-, TDN	Increases in EC, DOC and DON. Also incease in SO_4^{2-} of gravesoil suggested anaerobic respiration. Decrease in pH values of gravesoil. Increased nutrient movement of decomposition by-product off-site.	Aitkenhead-Peterson *et al.* (2012)
454 Pyrosequencing (NGS)	*In situ* aboveground	Soil epinecrotic bacterial community	Pig (*Sus scrofa domesticus*)	Temperature	Changes in bacterial community with increased activity during spring, summer and winter but decrease in autumn.	Pechal *et al.* (2013)
PCR, PCR-DGGE and cloning	*In situ* aboveground	Carcass necrophagous insect and bacterial community	Pig (*Sus scrofa domesticus*)	Temperature, relative humidity	Shift in necrophagous insect and bacterial communities observed from the mouth and rectum attributed to seasonal effects and carcass depletion.	Iancu *et al.* (2015)
qPCR, cloning, Sanger sequencing	*In situ* aboveground	Gut bacterial communities	Human cadaver	Not determined	Decrease in relative abundance of *Bacteroides* and *Lactobacillus*, as potential PMI indicator.	Hauther *et al.* (2015)

The fatty acids 18:2ω6 account numerically for about 43% of the total fatty acids of 47 soil fungal species (Kandeler, 2007).

Fatty acid methyl ester analysis is a rapid method used in community profiling for viable and non-viable microorganisms, and animal and plant biomass (Dunfield, 2007; Marschner, 2007). The method detected reliably changes in soil community profiles following agricultural management (Dunfield, 2007). Although the common fatty acids of animals, microorganisms and plants question the reliability of FAME in microbial community profiling, animal and plant fatty acids tend to be longer chained (C>20) than those of microorganisms (Dunfield, 2007; Marschner, 2007).

The successful application of PLFA and FAME (Hedrick *et al*., 2000; Marschner, 2007) in soil microbial profiling has prompted their adoption as forensic tools. This was demonstrated, for example, by Parkinson *et al*. (2009) where PLFA profiling of human cadaver gravesoil showed successional changes in the bacterial and fungal communities. Breton *et al*. (2016) used FAME to reveal that shifts in the fatty acid methyl esters profiles aligned with the start of active decay and continued until the dry period. Both seasonal and annual differences in the FAME profiles were recorded during the study, thus necessitating further investigation and validation for this technique to become an effective PMI estimation tool.

1.9.2 Nucleic acid-based community profiling

The development of non-culture techniques and their applications in polymerase chain reaction (PCR) amplification and hybridisation experiments have enabled researchers to identify and characterise numerous previously unidentified microorganisms (Muyzer *et al*., 1993; Thies, 2007). The use of nucleic acid extracts, as described by Thies (2007), falls into three categories:

(i) Direct analysis of extracted nucleic acids;

(ii) Analysis of PCR amplicons; and

(iii) *In situ* analysis of extracted nucleic acids.

Various techniques have been developed to monitor community DNA profile changes but the results are often simplified in comparison to the actual populations. Thus, according to Felske and Osborn (2004), community DNA profiling is defined by the specificity of the primers used for the amplification and the preferential DNA fragments. Commonly used techniques include denaturing- or temperature-gradient gel electrophoresis (DGGE, TGGE), T-RFLP and single strand conformation polymorphism (SSCP). While both DGGE and TGGE are based on DNA melting behaviour, T-RFLP generates DNA fragments due to the presence of restriction sites and SSCP separates microbial community members based on the melting behaviour of the secondary structure of single-stranded DNA (Felske and Osborn, 2004; Marschner, 2007; Madigan *et al*., 2012b).

As one of the common profiling tools, DGGE is a culture-independent molecular platform that has been used to analyse soil microbial fingerprints for changes in community diversity, composition and structure (Muyzer *et al*., 1993; Portillo *et al*., 2011; Madigan *et al*., 2012b). Analysis of microbial fingerprints by DGGE is based on the electrophoretic mobility of PCR amplicons of similar size but different base sequences that are denatured partially through a polyacrylamide gel of increasing concentrations of denaturants (usually formamide and urea) (Muyzer *et al*., 1993; Green *et al*., 2009; Madigan *et al*., 2012b). As reported widely (Muyzer *et al*., 1993; Green *et al*., 2009; Madigan *et al*., 2012b), the differences in electrophoretic mobility can be attributed to the guanine + cytosine (GC) contents of DNA fragments. According to Thies (2007), the stability of DNA is affected by both the GC content and the neighbouring nucleotide interactions. For example, a DNA sequence of GGA is more stable than that of GAG. The electrophoretic mobility of denatured DNA produces a series of bands, referred to as operational taxonomic units (OTUs) (Thies, 2007), characteristic of the PCR amplicons.

The application of DGGE as a possible forensic tool was demonstrated by Lerner *et al*. (2006) where the profiling of crime scene and alibi soils showed large bacterial diversity, which clustered according to soil type and location. Likewise, Parkinson *et al*. (2009) used T-RFLP and phospholipid fatty acid analysis, with relevant mathematical analyses, and showed soil bacterial and fungal community successional changes beneath two human cadavers that could be linked to their decomposition timelines, which were also cadaver specific *re* weight. According to Pasternak *et al*. (2012) both DGGE and T-RFLP were powerful techniques that distinguished successfully between soils and treatments with potential usage as forensic tools. Furthermore, the use of amplicon length heterogeneity-PCR, according to Moreno *et al*. (2011), produced distinct amplicons of anaerobic bacteria and variations in nitrogen-fixing bacteria that could be used to differentiate between control and gravesoils.

As reported by various researchers (Thies, 2007; Green *et al*., 2009; Crippen *et al*., 2015), DGGE has limitations which include: low sensitivity for detecting some rare community species; the possibility of two different species with similar melting properties appearing as a single band due to the GC contents of the DNA fragments; the restriction of amplicon sizes to under 600 base pairs making it difficult to recover full-length rRNA gene sequences; potential multiple bands derived from single organisms with multiple 16S rRNA gene operons; reporting diversity as OTUs; and inability to resolve community taxonomic classification (*i.e.*, phylum, class, order, family, genus and species).

Advances in molecular techniques have led to the creation and use of high throughput parallel sequencing tools referred to as next-generation sequencing (NGS) technologies, which can resolve complex environmental microbial communities (Crippen *et al*., 2015; Singh and Crippen, 2015). The most widely used tools include: Roche-454 pyrosequencing; Illumina (HiSeq/Miseq); and Ion-torrent (Allen and LaMontagne, 2015). Consequently, terms such as “microbiome”, “necrobiome” and “thanatomicrobiome” have been applied to

describe microbial communities associated with decomposition. According to Metcalf *et al*. (2013) and Pechal *et al*. (2014), successional changes in microbial community structure and composition can be used as a potential "postmortem microbial clock". Similarly, the work of Lauber *et al*. (2014) showed changes in the necrobiome of mice (*Mus musculus*) during active and advanced decay, while Carter *et al*. (2015) studied the ecological succession of soil microorganisms affected by decomposing swine carcasses and emphasized the importance of seasonality during PMI estimation. Also, Finley *et al*. (2016) observed changes in bacterial community composition with dominance of the Proteobacteria phylum for a human cadaver grave site.

Each technique incorporates nucleotides or oligonucleotides at the blunt end of each fragmented DNA strand. There are, however, some limitations of the various techniques as highlighted in Table 1.2.

Table 1.2: Advantages and disadvantages of various NGS platforms.

Sequence platform	Method	Approximate sequence length	Advantages	Disadvantages
Roche-454	Pyrosequencing	400 bp	High throughput, with longer sequence length.	High error rate because of homopolymers.
Illumina (HiSeq/MiSeq)	Sequencing by synthesis	150 bp	High throughput, lower error rate.	Short sequence length.
Ion-torrent	Semi-conductor sequencing technology	400 bp	High throughput, faster and cheaper cost per run.	High error rate because of homopolymers.

1.10 Research hypotheses

Against the above background and, specifically, key paucities in the literature, the following hypotheses were developed:

1. The subsurface decomposition of tissue and/or whole *S. scrofa domesticus* as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.
2. The subsurface decomposition of tissue and/or whole *S. scrofa domesticus* will effect different shifts in the surrounding soil microbiome structure and composition when compared to plant/leaf litter.
3. Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and plant/leaf litter decompositions.

To address these, three outdoor subsurface decomposition studies in perforated polyethylene containers (Study I – Study III) and a site (*in situ*) subsurface investigation (Study IV) were made. Study I investigated changes in soil microbial community biodiversity relative to animal tissue burial depth with time, while Study II compared changes in decomposition-driven biodiversity in response to tissue and plant litter burials with key environmental variables, such as temperature and pH, also measured. Study III explored the effects of thawed-from-frozen whole piglet decomposition and compared changes in soil biodiversity in the presence and absence of plant litter. Study IV was an *in situ* belowground decomposition study also with a whole animal carcass and leaf litter to mimic real crime scene clandestine burials in a forest environment. In recognition of the limitations of conventional physicochemical methods/parameters that are used typically in forensic decomposition studies, and the accepted strengths of molecular microbial ecology techniques [1.9; Table 1.1], this research programme employed denaturing gradient gel electrophoresis with attendant ecological index analysis, next-generation sequencing and relevant mathematical/statistical analyses methodologies. The aim was to assess the potentials of microbial ecology tools in (subsurface) decomposition studies to further knowledge development for enhanced PMI and PBI determinations. The ultimate goal of such programmes is to enhance the crime scene investigative toolkit, expand intelligence gathering in crime scenes and, ultimately, inform court proceedings.

Chapter 2: Materials and methods

2.1 Soil sources

Soils were sourced from four locations: **Soil I** (Study I) - a garden in Trimdon Station, County Durham, U.K. (Lat. 54.71°N, Long. 1.42°W); **Soil II** (Study II) - a well-secured site at Bishop Burton College of Agriculture, Lincolnshire, U.K. (Lat. 53.27°N, Long. 0.52°W); **Soil III** (Study III) - a well-secured site at Framwellgate Moor, County Durham, U.K. (Lat. 53.15°N, Long. 1.59°W); and **Soil IV** (Study IV) - a site located at Monk Fryston, North Yorkshire, U.K. (Lat. 53.76°N, Long. 1.23°W). The soils were transported in sterile 25 *l* air-tight buckets (Sarstedt, Germany). To ensure homogeneity, each was milled thoroughly (Retsch SM 100, Retsch, Haan, Germany) and sieved (ASTM - standard soil sieve N^{o} 10, 2 mm mesh; sterilised by autoclaving at 120°C, 15 psi for 20 min).

The impacts of decomposition on the microbial ecologies of different soil types are essential to investigate provided that the same animal model is used, as much as is practicable. Due to unavoidable and/or unforeseeable circumstances, this research programme consisted of four studies that used different soils and microcosm sizes. Specific challenges were faced and these included: (i) unapproved disposal of stored research soil; (ii) access to secure and approved space on the Teesside University campus; (iii) loss of communication with a supervisor's colleague after agreeing access to a study site and soil collection; (iv) fear of animal rights organisations in the U.K., which discouraged a land owner, who was contacted through the Northumbrian Police, to sanction decomposition research on his property; and (e) identification of a new site and extended negotiations for the *in situ* investigation.

2.2 Soil characterisation

The soils were subjected to physicochemical analyses (Forestry Commission, Surrey, U.K.; Derwentside Environmental Testing Services Ltd, Durham, U.K.) at the beginning of each

study for parameters highlighted by Spicka *et al*. (2011) and Cobaugh *et al.* (2015) (Table 2.1).

Table 2.1: Texture and physicochemical characteristics for Studies I – IV. ND denotes not determined.

	Soil I (Study I)	**Soil II (Study II)**	**Soil III (Study III)**	**Soil IV (Study IV)**
Clay (%)	ND	21	26	22
Silt (%)	ND	21	21	32
Sand (%)	ND	58	53	46
TOC (%)	2.8	5.9	4.1	3.0
Aqueous NO_3^- (mg l^{-1})	1.5	76	4.6	3.5
Total S (%)	0.03	0.05	0.03	0.03
Ortho PO_4^- (mg kg^{-1})	<0.01	1.0	1.2	<0.10
pH	7.6	5.8	6.3	7.9

2.3 Carbon sources

Although different animal models such as mouse (*Mus musculus*), rat (*Rattus rattus*), sheep (*Ovis aries*) and Dawley rats have been used (*e.g.* Carter and Tibbett, 2006; Lauber *et al*., 2014; Guo *et al*., 2016), domestic pig (*Sus scrofa domesticus*) is largely accepted as the most appropriate human taphonomic proxy in decomposition studies (Van Belle *et al*., 2009; Schotsman *et al*., 2011) for enhanced PMI and time-since-burial determinations [Table 1.1]. Therefore, leg and freshly butchered pig meat were bought from a local butcher (Middlesbrough, U.K.) and washed thoroughly with deionised water (Arium®Advance, Sartorius, Germany). The former was used whole for the preliminary non-replicated Study I while the latter was cut into similar 4 g cubes to facilitate a triplicated and destructively sampled experimental design in Study II. Still-born piglets were sourced from the

Northumbria Police (Ponteland, U.K.) and used for Study III (non-replicated) and Study IV to mimic criminal burials, in triplicate, of a whole body. All the pig muscle tissues and stillborn piglets were sourced from an organic butcher and organic farm, respectively.

To reflect plant litter materials that are likely to be incorporated in clandestine burials, freshly mown grass litter (*Agrostis*/*Festuca* spp) was collected from a domestic garden (Wynyard, U.K.) (Study II and Study III). Leaf litter (*Quercus robur*) from the surrounding deciduous forest was used for the *in situ* experiment (Study IV).

2.4 pH

Soil samples from every sampling time of all four studies were each mixed thoroughly with sterile deionised water in a ratio of 1:5 (w/v) and their pH measured according to the method described by Stokes *et al*. (2009) with a pH probe (Fisher Scientific, Loughbrough, U.K.) and 213 microprocessor (Hanna Instruments, Bedfordshire, U.K.).

2.5 Temperature

Soil temperatures were measured with a hand-held Hanna thermometer (Hanna Instruments, Bedfordshire, U.K.) at every sampling time, while atmospheric temperatures for Middlesbrough and Monk Fryston were obtained from http://www.metoffice.gov.uk/.

2.6 Soil DNA extraction

Extractions of total soil microbial DNA were made with the FastDNA®Spin Kit (Knauth *et al*., 2013; Pakpour *et al*., 2013; Finley *et al*., 2015) for Soil (MP Biomedicals, U.K.) according to the manufacturer's instructions. Thus, soil samples (500 mg) were added to the Lysing Matrix E tubes followed by 978 µ*l* of sodium phosphate buffer and 122 µ*l* MT buffer. Cell lysis was achieved by homogenisation in a Precellys 24 lyser homogenizer (Bertin Technologies, France) with the following variables: run time (23 seconds); speed (6 500 rpm); number of cycles (2); and break between cycles (5 seconds), followed by centrifugation (Sigma, Harz, Germany) at 14 000 × g for 7 minutes. The supernatants were

transferred into sterile 2 m*l* microcentrifuge tubes (Sarstedt, Germany) and protein precipitation solution (250 µ*l*) added before thorough shaking (10 times). The mixtures were centrifuged (Sigma, Harz, Germany) at 14 000 × g for 5 minutes and the supernatants transferred to clean sterile 2 m*l* microcentrifuge tubes. Re-suspended binding matrix solution (1 m*l*) was added and the tubes inverted by hand for 2 minutes with the matrix then allowed to settle at room temperature for 3 minutes. Supernatant aliquots (500 µ*l*) were discarded with the remaining solutions (*ca* 600 µ*l*) re-suspended and transferred into SPIN^{TM} filters and centrifuged (Sigma, Harz, Germany) at 14 000 × g for 1 minute. The catch tubes were emptied and the remaining mixtures were added to the SPIN^{TM} filters and centrifuged as before. The catch tubes were emptied again and 500 µ*l* freshly prepared SEWS-M added gently to re-suspend the pellets on the SPIN^{TM} filters. These were then centrifuged (Sigma, Harz, Germany) at 14 000 × g for 1 minute, the catch tubes emptied, replaced and centrifugation repeated at 14 000 × g for 2 minutes. The catch tubes were discarded and replaced by clean sterile tubes. The SPIN^{TM} filters were air dried for 5 minutes at room temperature and 100 µ*l* of DES (DNase/Pyrogen – free water) were added to re-suspend the Binding Matrix on the SPIN^{TM} Filters. These were then incubated (Grant, Shepreth, U.K.) at 55°C for 5 minutes and centrifuged (Sigma, Harz, Germany) at 14000 × g for 1 minute to elute the DNA extracts, which were then stored at -20°C until required.

2.7 DNA purification of samples for NGS

For next-generation sequencing, the extracted DNA samples were purified with PowerClean® DNA Clean-Up Kit (Mo Bio Laboratories, Inc., U.S.A.) according to the manufacturer's instructions. Thus, for each purification 50 µ*l* of the DNA extract were transferred into a sterile 2 m*l* microcentrifuge tube (Mo Bio Laboratories, Inc., U.S.A.) and the volume increased to 150 µ*l* with the addition of DNase/RNase-free water (Fisher Scientific, U.S.A.). PowerClean® DNA solution 1 (70 µ*l*) was added and the solution mixed gently. PowerClean® DNA solution 2 was pre-heated (Grant, Shepreth, U.K.) to 60°C and

20 µ*l* added. A further 85 µ*l* of PowerClean® DNA solution 3 were added with gentle mixing before incubation at 4°C for 5 minutes. The DNA solution was then centrifuged (Sigma, Harz, Germany) at 10 000 × g for 1 minute. The supernatant was transferred into a sterile 2 m*l* microcentrifuge tube (Mo Bio Laboratories, Inc., U.S.A.) and 70 µ*l* of PowerClean® DNA solution 4 added before gentle mixing and incubation at 4°C for 5 minutes followed by centrifugation (Sigma, Harz, Germany) at 10 000 × g for 1 minute. The supernatant was again transferred into a sterile 2 m*l* microcentrifuge tube (Mo Bio Laboratories, Inc., U.S.A.) and 500 µ*l* of PowerClean® DNA solution 5 added. The solution was vortexed for 5 seconds after which 600 µ*l* were transferred onto a Spin filter and centrifuged (Sigma, Harz, Germany) at 10 000 × g for 1 minute. The flow through was discarded and the remaining solution (*ca* 600 µ*l*) transferred into a Spin filter and centrifuged (Sigma, Harz, Germany) at 14 000 × g for 1 minute. PowerClean® DNA solution 5 (500 µ*l*) was added to the filter which was then centrifuged (Sigma, Harz, Germany) at 10 000 × g for 30 seconds. The catch tube was emptied, replaced and the centrifugation repeated at 13 000 × g for 1 minute. The catch tube was again discarded and replaced by a clean sterile tube. PowerClean® DNA solution 7 (50 µ*l*) was then added to the Spin filter before centrifugation (Sigma, Harz, Germany) at 10 000 × g for 30 seconds to elute the DNA extract, and storage at -20°C until further use.

2.8 Agarose gel electrophoresis

DNA samples (5 µ*l*) were mixed with 1 µ*l* of 6X loading buffer (Bioline, U.S.A.), checked on 1.5% (w/v) agarose (Fisher Scientific, U.S.A.) gels prepared with 1X TBE buffer (Mo Bio Laboratories, Inc., U.S.A.) and stained with 6 µ*l* of SYBR Safe (Invitrogen, U.S.A.). The gels were electrophoresed in 1X TBE buffer for 90 minutes at 150 V and viewed under UV light with an AlphaImager HP® (Alpha Innotech, Braintree, U.K.).

2.9 Polymerase chain reaction conditions

Amplification of the DNA extracts was made with a Primus 96 Plus thermocycler (MWG-Biotech) and Promega master mix which contained initial concentrations of *Taq* DNA

polymerase (50 U ml^{-1}), dATP (400 µM), dGTP (400 µM), dCTP (400 µM), dTTP (400 µM), $MgCl_2$ (3 mM) and BSA (10 mg ml^{-1}). The final reaction volumes were 25 µ*l* and included 2 µ*l* of template DNA. Primer sets were used to amplify the bacterial 16S rRNA (GC388F/530R) (Manefield *et al.*, 2002) and fungal 18S rRNA (NS1/NS8 then NS1/NS10) (White *et al.*, 1990) genes. The amplification conditions for each are shown in Table 2.2. Both positive (*E. coli*) and negative controls (nuclease free-water) were amplified parallel to the studies' DNA extracts to determine the presence of contaminants.

Table 2.2: Primer pairs and thermocycle programmes used for DNA amplification.

Primer pairs	Final primer conc^n (µM)	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
GC388F/ 530R	0.2	95°C 2 min	95°C 1 min	60°C 1 min	72°C 1 min 30 sec	72°C 30 min	35
NS1/NS8	1	94°C 2 min	94°C 30 sec	50°C 45 sec	72°C 2 min	72°C 2 min	30
NS1/NS10	1	94°C 2 min	94°C 30 sec	55°C 1 min	72°C 1 min 15 sec	72°C 5 min	35

2.10 DGGE separation of PCR amplicons

The PCR amplicons (15 µ*l*) for the bacterial and fungal communities were each mixed with 3 µ*l* of 6X loading dye (Bioline, U.S.A.) and separated on 10% (w/v) and 6% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide gel 37.5:1) with 30 - 65% and 25 - 45% denaturing gradients, respectively. The gels were cast and run with a DCode™ universal mutation detection system (Biorad, Hertfordshire, U.K.) at 60°C and 110 V for 16 hours. They were then stained with SYBR Gold (Invitrogen) and viewed under UV light with an AlphaImager HP® (Alpha Innotech, Braintree, U.K.).

2.11 DGGE image analysis

The DGGE images were analysed with Phoretix 1D software (TotalLab, Newcastle, U.K.) for quantification of the community fingerprints (Wang *et al*., 2011; Adewumi *et al*., 2013). The lanes and bands of each were detected automatically. The number of bands in each lane was determined and a matrix of bands intensities created. Background noise was subtracted by using the rolling-ball algorithm with a radius of 50 pixels. Similarities between lanes were determined by boundary identification, band detection and creation of a synthetic reference lane. The relative abundance of each band was defined as the ratio of its intensity to the total intensity of the lane for each sample. Each band was assumed to be a unique phylotype for a bacterial species and referred to as an operational taxonomic unit (OTU) (Dos Santo *et al*., 2009). The number of bands (OTU/species) and their relative abundances (band intensity) were used to estimate the diversity of the community (Nubel *et al*., 1999; Hedrick *et al*., 2000; Dos Santo *et al*., 2009). Shifts in community structure from detectable bands/OTUs were assessed with ecological indices measures of richness (*S*) and diversity (Shannon-Wiener, *H′*, Equation 2.1; Simpson, *D*, Equation 2.2) (Hill *et al*., 2003; Lamb *et al*., 2009; Bandeira *et al*., 2013).

Diversity can be defined as the measure which reflects species richness and relative abundance in a community, which permits the evenness of the community to be compared (Nubel *et al*., 1999; Lamb *et al*., 2009; Bandeira *et al*., 2013). According to Bandeira *et al*. (2013), the use of a single ecological index to define a community overgeneralises its actual diversity so the use of different indices to study diversity is recommended. The Shannon-Wiener index, as described by various researchers (Nubel *et al*., 1999; Strien *et al*., 2012; Barry *et al*., 2013), is a function of evenness and species relative abundance, while the Simpson index is a function of richness and relative abundance.

$$\text{Shannon-Wiener index } (H') = -\sum \boldsymbol{P}_i \ln \boldsymbol{P}_i \qquad \text{Equation 2.1}$$

$$\text{Simpson index } (D) = 1 - \sum Pi^2 \qquad \text{Equation 2.2}$$

2.12 Data analysis

All data were tested with Shapiro-Wilk W for normal distribution prior to analysis. The soil pH, temperature and ecological indices were evaluated statistically by a univariate two-way ANOVA with repeated measure (RMA) where F statistic is the variance of group means divided by mean of within group variances. To ensure robustness beyond the overall mean used in ANOVA, specific temporal statistical differences between each treatment and control were determined by further analysis with the Tukey (HSD) *post hoc* test (Xlstats 2016.02.27313, New York, U.S.A.). Relationships between the ecological measures were assessed further with principal component analysis (Xlstats 2016.02.27313, New York, U.S.A.).

For NGS data, the phylogenetic distance matrices were analysed by Bray-Curtis dissimilarity with nonmetric dimensional scaling (NMDS) by the paleontological statistics software package for education and data analysis (PAST 3.10, 2015). Alpha diversity was estimated with Shannon diversity (Gonzalez *et al*., 2011; Pylro *et al*., 2014), which was expressed by boxplot with xlstats. Relationships between soil pH, temperature and phyla relative abundance were analysed using Spearman's rank correlation coefficient (SCC) (xlstats 2016.02.27313, New York, U.S.A.) where **R** is the correlation coefficient. Taxa with relative abundances >3% were plotted with Microsoft Excel (Microsoft 2013). To identify distinct taxa, heatmaps were constructed using the gplots package (R version 3.3.2, gplots version 3.0.1; R Core Team). Differences between controls and treatments were analysed by PERMANOVA (PAST 3.10, 2015). To reveal significant differences in OTU between controls, treatments and season, pair wise multiple comparisons after a multi-way ANOVA with Tukey (HSD) *post hoc* test ($p < 0.05$) were used (Cobaugh *et al*., 2015; Singh *et al*., 2017; Thomas *et al*., 2017).

Chapter 3: Changes in soil microbiome communities: A preliminary decomposition study [2]

3.1 Introduction

A microcosm is an (artificial) simplified ecosystem that is used to study ecological processes in a controlled environment. Microcosm-based study is a well-established procedure that has been used in high-impact forensic research for decomposition knowledge development (Carter *et al.*, 2010; Bergmann *et al.*, 2014). For example, Tibbett *et al.* (2004) studied skeletal muscle decomposition at (12 and 22˚C) and reported a higher muscle loss and microbial respiration at 22˚C when compared to incubation at 12˚C. Likewise, Carter and Tibbett (2006) observed decomposition rate changes at different temperatures (2, 12 and 22˚C) while Stokes *et al.* (2009) reported that freezing had no significant effect on decomposition rate when compared to unfrozen skeletal muscle in a controlled microcosm study.

Forensically, soil is regarded as important crime scene evidence (Petraco *et al.*, 2008; Woods *et al.*, 2014) and is subjected, typically, to physicochemical analyses such as carbon dioxide emission, ninhydrin analysis and organic matter determination (Carter and Tibbett, 2006; van Belle *et al.*, 2009; Damann *et al.*, 2012). In contrast, the efficacies of biochemical and microbiological profiling, particularly in decomposition/burial soils, have been recognised only relatively recently (Metcalf *et al.*, 2013; Hyde *et al.*, 2015). Various factors, including soil horizon and/or depth, electron donor/acceptor type and occurrence, nutrient availability, temperature, moisture content, redox potential, *etc.*, determine soil heterogeneity and, hence,

[2] A substantive proportion of this work was published as follows:

Olakanye A.O., Thompson T.J.U. and Ralebitso-Senior, T.K. 2014. Changes to soil bacterial profiles as a result of *Sus scrofa domesticus* decomposition. *Forensic Science International* **245**: 101–106.

Chimutsa M., Olakanye A.O., Thompson T.J.U. and Ralebitso-Senior T.K. 2015. Soil fungal community shift evaluation as a potential cadaver decomposition indicator. *Forensic Science International* **257**: 155–159.

the physiological, phenotypic and phylogenetic diversities of its microbial communities. As in other microbial ecology contexts, understanding the roles of microbial community parameter changes during decomposition is essential for subsequent application in real crime scenes.

As discussed earlier [1.9], the importance of cadaver decomposition knowledge is reflected by emergent studies that have analysed changes in the postmortem microbiomes or necrobiomes of the abdominal, interior anal and buccal cavities and skin of decomposing carcasses (Hyde *et al*., 2013; Metcalf *et al*., 2013; Pechal *et al*., 2014). Nonetheless, the majority of research has focused on aboveground decomposition (Campobasso *et al*., 2001; Stokes *et al*., 2009; Howard *et al*., 2010). As a result, considerable knowledge gaps remain of soil microbial community changes following subsurface cadaver decomposition (Carter and Tibett, 2006; van Belle *et al*., 2009; Carter *et al*., 2010). Determining the relationship between soil ecology and cadaver decomposition as a tool for accurate PMI estimation can be very challenging. To gain a better understanding of this relationship, this preliminary study was made and analysed by PCR-DGGE and NGS to test the programme hypothesis 1 with the principal objective of assessing, (i) temporal soil community changes relative to burial depth of *Sus scrofa domesticus* (PCR-DGGE); and (ii) bulk sample analyses between the control and *S. scrofa domesticus* treatment to determine microbial community composition (NGS).

3.2 Experimental design

Two polyethylene containers with 20 kg (fresh weight) sandy loamy garden soil (Soil I) were maintained outdoors (Teesside University, Middlesbrough, U.K.; Lat. 54.5722°N, Long. 1.2349°W) for 98 days at ambient temperature with (experimental) and without (control) a 5 kg pig (*Sus scrofa domesticus*) leg. The containers were 40 cm in height and divided into four sections (empty, 0 – 10 cm; top, 10 – 20 cm; middle, 20 – 30 cm; and bottom, 30 – 40 cm) and were perforated vertically every 5 cm and horizontally every 7 cm, and the bottom

and lid for hygiene maintenance, sampling, aeration and moisture migration. Soil samples were collected from the three sections through the peforations with disposable sterile probes on days 0, 3, 7, 10 and 14 and then weekly until day 98. Composite soil samples (5 g) were collected via the perforations from each section every three days for the first week, and then weekly for the study duration. The samples were stored (25 m*l* sterile universal bottles; Sarstedt, Germany) at -20°C until required for DNA extraction [2.6].

The average daily temperature of 14.9°C and precipitation of 3.5 mm were recorded during the study with day 28 registering the highest temperature of 18.1°C and rainfall of 13.5 mm (metoffice.gov.uk).

3.3 DNA extracts for next-generation sequencing

All DNA templates from each sampling time and soil sections were subjected to DNA purification [2.7], then pooled to facilitate comparisons of total microbial community structures between the soil control and *S. scrofa domesticus* treatment.

The samples were sequenced with Illumina Miseq platform (Research and Testing Laboratory, Lubbock, Texas, U.S.A.) with the primer sets 28F/519R (16S bacterial gene; V1 – V3 region; 5'-GAGTTTGATCNTGGCTCAG-3'/5'-GTNTTACNGCGGCKGCTG-3') (Research and Testing Laboratory, Lubbock, Texas, U.S.A.; Fan *et al*., 2012). The raw sequences were processed in FASTQ format, merged with PEAR Illumina paired-end read merger and converted into FASTA formatted sequences for quality checking and filtering. Operational taxonomic unit selection was made with UPARSE and Chimera checking was performed with UCHIME executed in *de novo* mode. Taxonomy was assigned by USEARCH global search algorithm (http://drive5.com/usearch/) while the phylogenetic tree was constructed with MUSCLE (www.researchandtesting.com/"version 2.2.4"). PCR negative controls were run and sequenced in parallel to the samples with OTUs present in negative controls and samples excluded from further analysis. OTUs less than 3% were

classified as rare taxa. Both the rare taxa and the unclassified OTUs were omitted from the plots.

3.4 Data analysis

Ecological indices [2.11] were used to analyse the DGGE community fingerprints. The data were tested with Shapiro-Wilk W for normal distribution prior to analysis. One-way ANOVA was used to determine differences between specific sections of the control and *S. scrofa domesticus* ($p < 0.05$) treatments. All data were subjected to the Palaeontological software package for education and data analysis (Past 3.10).

3.5 Results

3.5.1 Physical observations

Visual examination of the top section of the *S. scrofa domesticus* treatment revealed that active decay occurred between days 10 and 56. Adipocere formation was observed on day 42 in conjunction with a 4 cm top section soil collapse compared with the control (Figure 3.1).

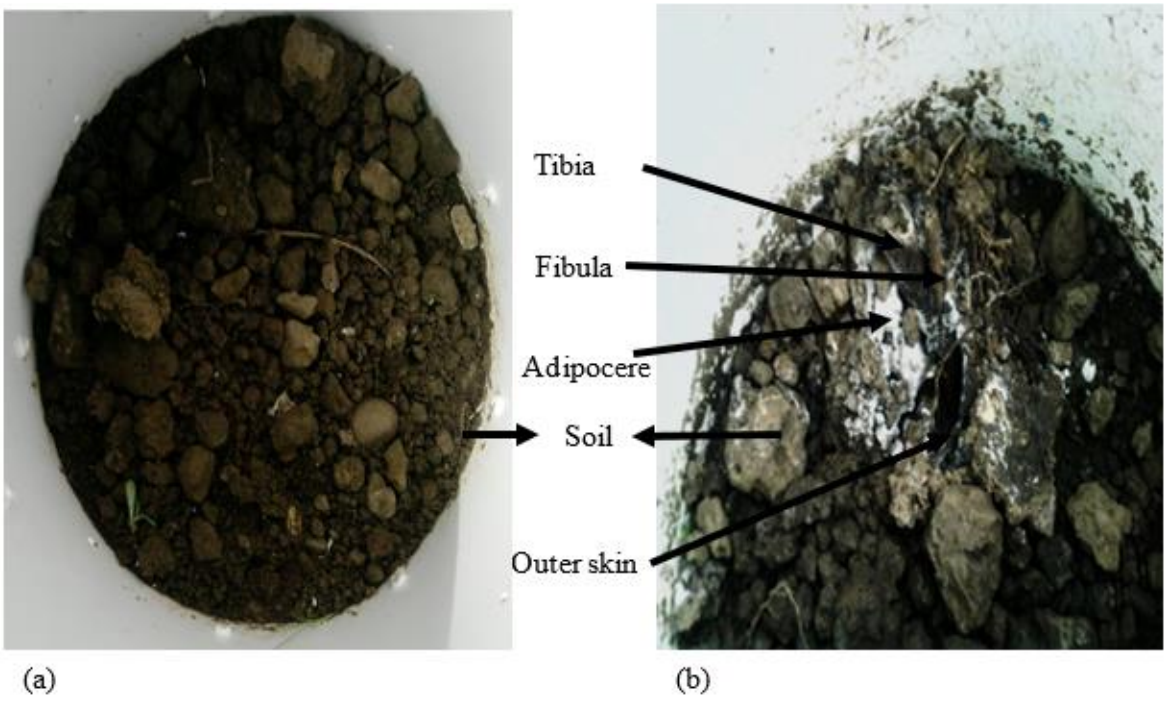


Figure 3.1: Day 42 surface views of the control (a) and pig (b) treatment.

By day 56 the pig leg soft tissues had decomposed completely with the tibia and fibula revealed. The void space created by the decomposition was filled partially with soil and water while maggot and insect activities were also apparent. Also, the skin had formed a barrier against the soil. The order of tissue decomposition, as reported by Gill-King (1997), indicates that the high collagen contents of the integumentary system components (exocrine glands, hair, nails and skin) usually makes them the last to decompose.

3.5.2 DGGE profile analysis

Soil ecological analysis

Richness

The DGGE fingerprints were analysed with ecological indices [2.11] to estimate the shifts in microbial communities in the presence or absence of *S. scrofa domesticus*. Species richness (S) was calculated by the number of visible bands (OTUs) in each lane. No statistically significant difference was recorded for the species richness between the top ($p = 0.389$) and middle ($p = 0.442$) sections in the presence or absence of *S. scrofa domesticus*, while there was a mathematically significant difference between the control and *S. scrofa domesticus* treatment for the bottom section ($p = 0.007$) (Figure 3.2).

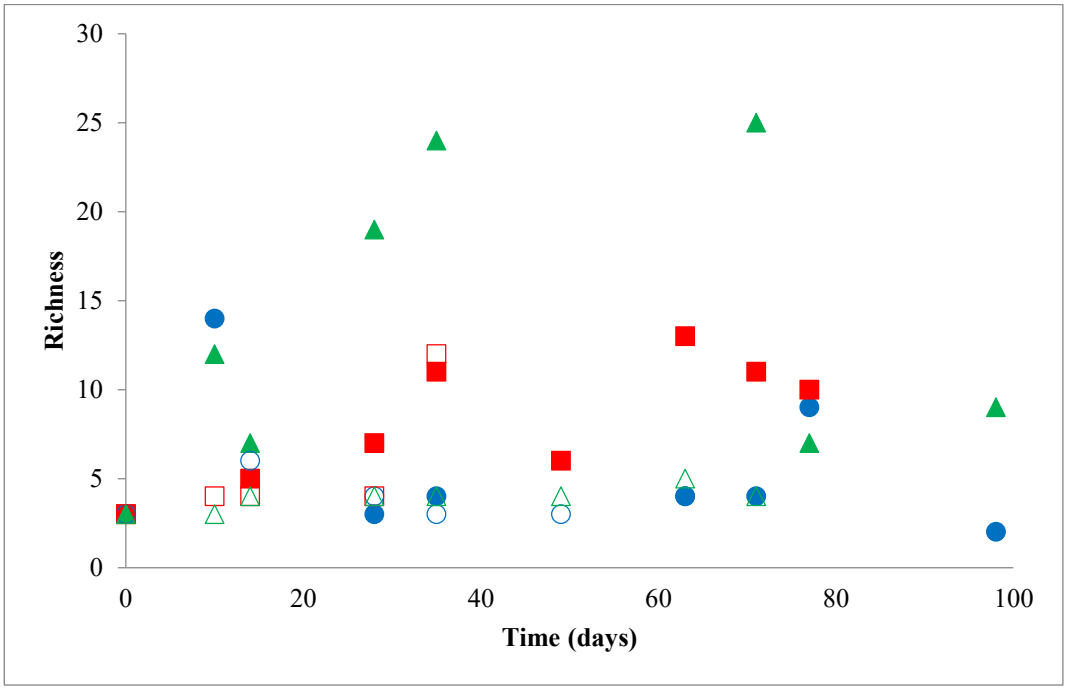


Figure 3.2: Richness of the control (top ○, middle □, bottom Δ) and *S. scrofa domesticus* (top ●, middle ■, bottom ▲) treatment.

The cumulative richness values of sections of the control (top, 23; middle, 27; bottom; 31) and *S. scrofa domesticus* (top, 43; middle, 66; bottom, 106) treatment also showed statistically significant differences ($p < 0.05$).

Shannon-Wiener diversity

As recorded for community richness, there was no significant difference between the control and *S. scrofa domesticus* treatment for their top ($p = 0.538$) and middle ($p = 0.136$) soil sections while there was for their bottom layers ($p = 0.0015$) particularly from day 28. Furthermore, a significant difference was observed between the different depths in the presence of the *S. scrofa domesticus* treatment ($p = 0.032$) but not for the control soil ($p = 0.46$). In general, an average of the Shannon-Wiener diversity measurements for the top, middle and bottom layers for the control and burial soils showed a significant difference ($p = 0.00004$) between the total communities due to the presence of *S. scrofa domesticus* (Figure 3.3).

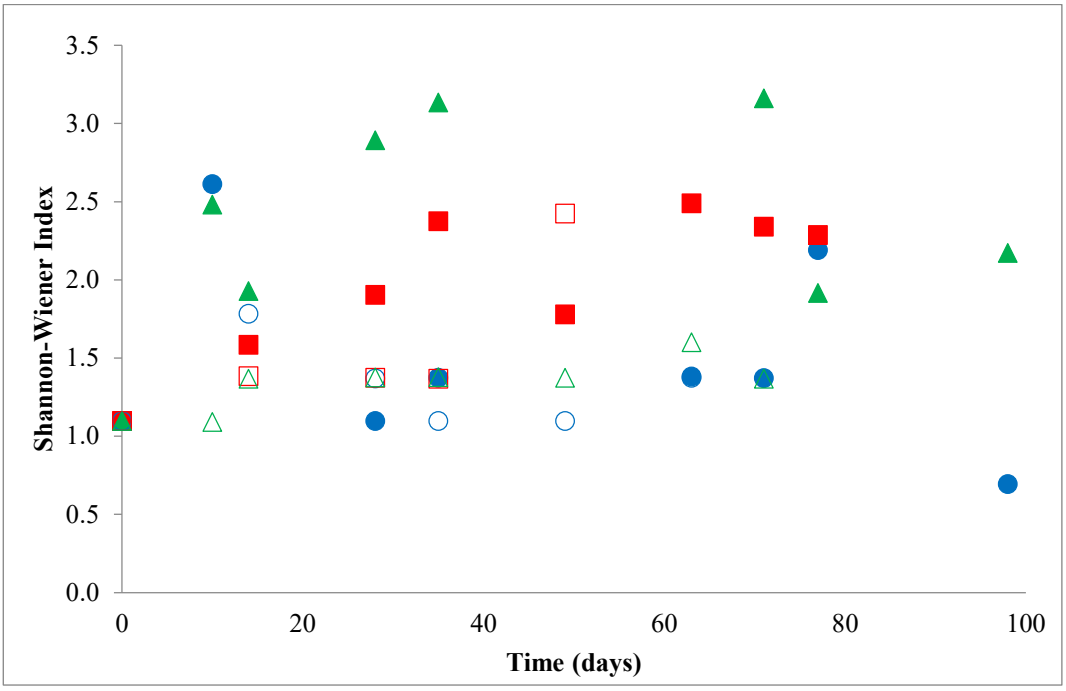


Figure 3.3: Shannon-Wiener diversity index (*H'*) of the control (top ○, middle □, bottom △) and *S. scrofa domesticus* (top ●, middle ■, bottom ▲) treatment.

Simpson diversity

The Simpson diversity index showed a significant difference between the control and *S. scrofa domesticus* treatment for the bottom sections (p = 0.0057). In contrast, but just as measured with the Shannon-Weiner index, no significant differences were recorded for the top (p = 0.796) and middle (p = 0.122) layers when comparing the control and *S. scrofa domesticus* treatment (Figure 3.4). Overall, an average of the Simpson diversity measurements for the top, middle and bottom layers showed a significant difference (p = 0.000029) between the total communities as a result of *S. scrofa domesticus*.

3.5.3 NGS 16S Taxonomic resolution

Phylum-level resolution

A total of 43 918 sequences was obtained for the control and *S. scrofa domesticus* treatment across 17 phyla with Proteobacteria (37.2 – 40.2%) the most dominant for both. Other dominant phyla included: Actinobacteria (23.2 – 25.3%); Acidobacteria (6.6 – 16.8%);

Firmicutes (7.9 – 8.9%); Bacteroidetes (2.2 –11%); and Chloroflexi (2.3 – 4.1%) (Figure 3.5).

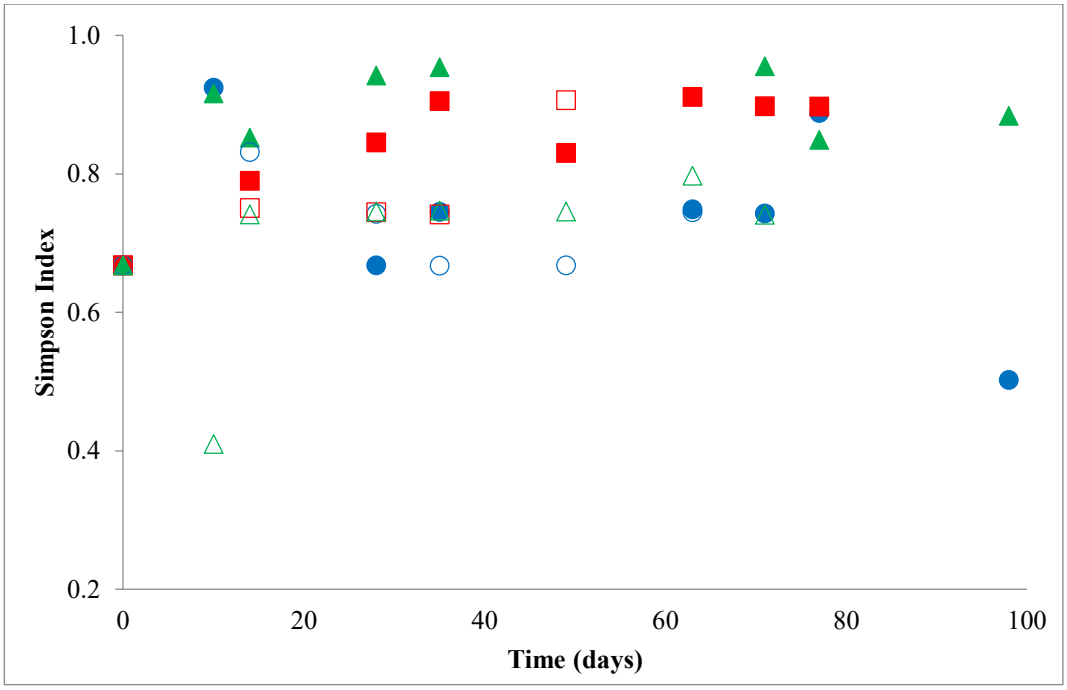


Figure 3.4: Simpson diversity index (D) of the control (top ○, middle □, bottom Δ) and *S. scrofa domesticus* (top ●, middle ■, bottom ▲) treatment.

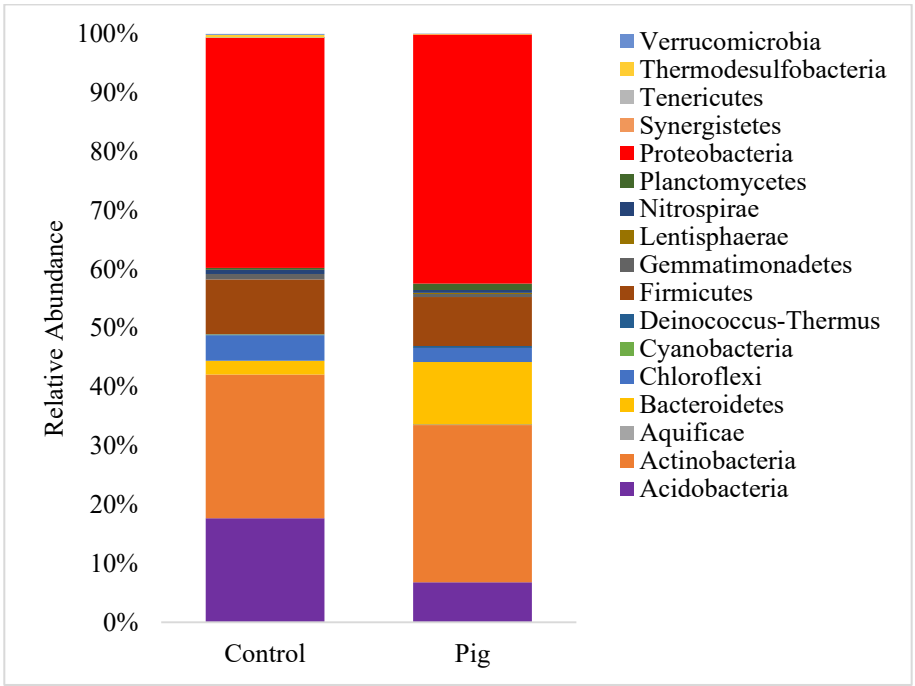


Figure 3.5: Phylum-level bacterial taxa resolution of pooled DNA samples of the control and *S. scrofa domesticus* treatment.

Order-level resolution

Order level taxonomic resolution (Figure 3.6) identified changes in abundance between the control and *S. scrofa domesticus* treatment with Acidobacteriales and Rhizobiales dominances recorded for the control while Xanthomonadales, Flavobacteriales, Sphingobacteriales and Pseudomonadales dominances resulted for the *S. scrofa domesticus* treatment.

Family-level resolution

Taxonomic resolution at family level (Figure 3.7) revealed changes in abundance between the control and *S. scrofa domesticus* treatment. Specifically, Acidobacteriaceae, Bacillaceae and Hyphomicrobiaceae dominances were recorded for the control and Microbacteriaceae, Streptomycetaceae, Flavobacteriaceae, Sphingobacteriaceae, Moraxellaceae, Pseudomonadaceae and Xanthomonadaceae dominances for the *S. scrofa domesticus* treatment.

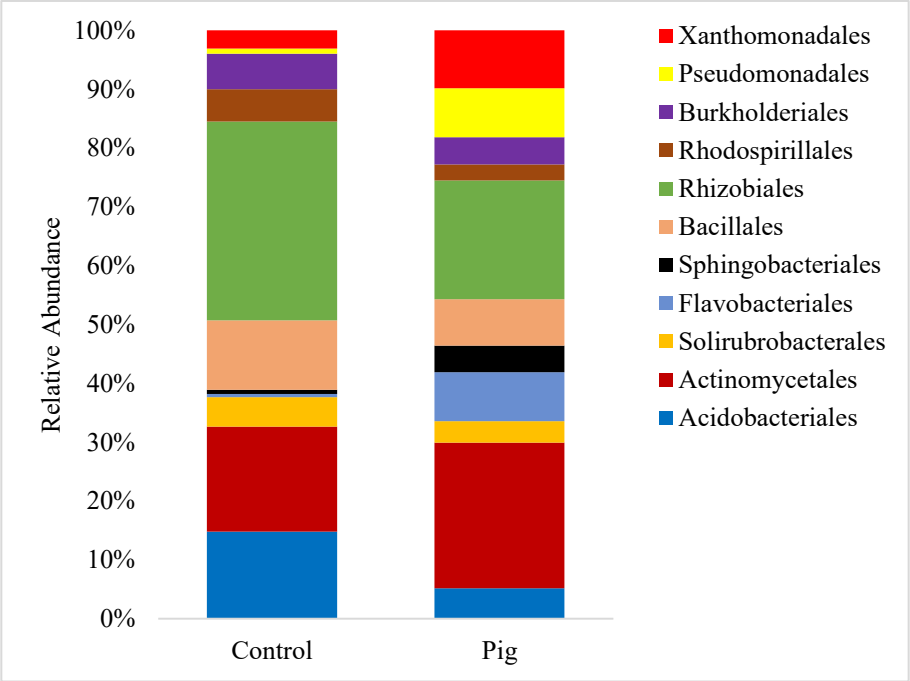


Figure 3.6: Order-level bacterial taxa resolution of pooled DNA samples of the control and pig (*S. scrofa domesticus*) treatment.

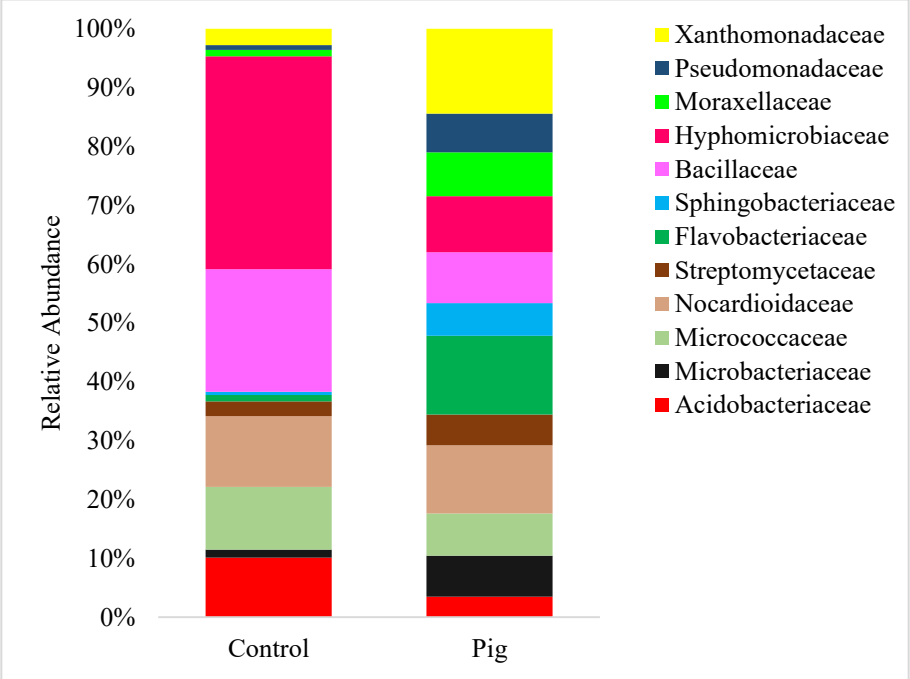


Figure 3.7: Family-level bacterial taxa resolution of pooled DNA samples of the control and pig (*S. scrofa domesticus*) treatment.

3.6 Discussion

Physical observations

The rate of decomposition, as described by various scholars (Vass *et al*., 2002; Forbes 2008; Janaway *et al*., 2009), is influenced largely by the means of body disposal, *i.e.*, dumped, buried, frozen, submerged, which subsequently affect its interactions with the fauna, flora and microbiota of its surroundings. As reported by Belle *et al*. (2009), the active decay stage for a belowground cadaver occurs within a period of three months. This was supported in the current research where the active decay period, as evidenced by extensive tissue decomposition, was recorded between days 10 and 42. On day 56 the soft tissue of the *S. scrofa domesticus* leg had decomposed completely, thereby creating a void between the tibia, fibula and skin, with the skin forming a barrier against the sandy loamy soil. According to Gill-King (1997), in the order of tissue decomposition, connective tissue and integuments are usually the last to decompose because of their high collagen contents. Again, this was in agreement with observations of the *S. scrofa domesticus* treatment on day 56.

Another well reported parameter of cadaver decomposition is the formation of adipocere (Forbes 2008; Gun 2009; Schotsmans *et al*., 2011; Schoenen and Schoenen 2013), which was observed on day 42 and is considered to be from bacterial activities in a warm, moist and anaerobic environment (Forbes 2008; Janaway *et al*., 2009; Schoenen and Schoenen 2013). According to Ubelaker and Zarenko (2011), adipocere is an essential taphonomic phenomenon due to its preservative characteristics of soft tissues, and ability to reveal constitutional and environmental factors that may prove valuable in forensic investigation with its complicating role in the determination of postmortem interval due to its preservative characteristics.

DGGE-based ecological indices

Soil microbial profiling at various depths in a grave soil can be essential to understand and estimate changes in microbial communities as a potential forensic tool. As observed in this study, different depths in the control and *S. scrofa domesticus* treatment gave an insight of how microbial communities change with both the decomposition timeline and the position/depth of the buried material. These changes can be attributed to vertical/gravitational leaching of nutrients in the *S. scrofa domesticus* treatment when compared to the control. The PCR-DGGE analysis showed changes in the community profiles over the course of decomposition from days 10 to 77 with some distinct numerically dominant OTUs recorded for the top, middle and bottom sections when compared to the control.

The diversity trends in the presence of decomposing tissue clearly showed a significant difference ($p < 0.05$) between the top, middle and bottom sections, with the top section having the lowest total cumulative richness value of 43, the middle section recording a cumulative richness value of 65 and the bottom section registering the highest cumulative value of 106. Also, as suggested by McGuire and Treseder (2010), a rise in microbial species richness is linked to an increase in decomposition. This was observed for the three sections

of the *S. scrofa domesticus* treatment with the bottom section, in particular, recording the highest increase in richness from 11 (day 10) to 26 (day 70).

According to Bandeira *et al*. (2013), the use of a single ecological index to define a community overgeneralises its actual biodiversity so the use of different indices to study diversity is recommended. The Shannon-Wiener index, as described by various scholars (Strien *et al*., 2012; Bandeira *et al*.; 2013; Barry *et al*., 2013), is a function of evenness and species relative abundance, while the Simpson index is a function of richness and relative abundance. Despite the use of different, if complementary, ecological measurements of the bacterial community dynamics, H' and D still showed similar trends of significant differences in diversity for the three sections of the *S. scrofa domesticus* treatment when compared to the control. This suggested a vital role of sample collection and subsequent community profiling from clandestine grave soils relative to the position or depth of the buried material. Furthermore, both indices showed distinct differences in bacterial community diversity in the absence and presence of *S scrofa domesticus*. Therefore, DGGE profiling is a possible tool to differentiate between grave and non-grave soils.

Microbial community taxonomic resolution

Cadaver decomposition is a complex process that affects the soil microbiota. The ability to identify components that are involved actively in and impacted by decomposition, irrespective of the cadaver microbiome, justifies further the relevance and applicability of cadaver/soil ecology interaction analyses for postmortem interval and time-since-burial estimations. For example, the transition from aerobic to anaerobic bacteria (*Bacteroides, Clostridium, Streptococcus, Staphylococcus* spp and Enterobacteriaceae group) during putrefaction has been reported widely by various researchers (Carter *et al*., 2010; Damann and Carter 2013). This triggers the breakdown of lipids, carbohydrates and proteins into organic acids (*e.g.* butyric acid, lactic acid, propionic acid) and gases (*e.g.* ammonia, methane, sulphur dioxide, hydrogen sulphide), which results in intense odour, colour change

and swelling of the cadaver (Campobasso *et al*., 2001; Vass *et al*., 2002; Forbes 2008). Furthermore, the seepage of these by-products changes the microbiota and soil chemistry temporarily (Moreno *et al*., 2011).

The use of next-generation sequencing techniques in microbiome studies as a potential PMI tool has aided the identification of microbial taxa that are involved in decomposition (Lauber *et al*., 2014; Pechal *et al*., 2014; Hyde *et al*., 2015). According to Metcalf *et al*. (2016), approximately 40% of the total soil microbiota are involved at the onset of decomposition at low relative abundance. Although common to most soils, some identified dominant bacterial phyla have been reported in forensic contexts (Hyde *et al*., 2013; Cobaugh *et al*., 2015) and include: Proteobacteria; Actinobacteria; Bacteroidetes and Firmicutes. These were consistent with this study where they accounted for approximately 90% of the bacterial phyla.

Analysis of the bulk samples revealed specifically the dominances of Hyphomicrobiaceae, aerobic Gram-negative chemoorganotrophic rod-shaped denitrifying bacteria (Mills *et al*., 2008) and Bacillaceae, Gram-positive aerobic or facultative anaerobic chemoorganotrophic rod-shaped bacteria (De-Vos *et al*., 2009), for the control soil while Sphingobacteriaceae, aerobic rod-shaped bacteria (Krieg *et al*., 2010) and Xanthomonadaceae, obligate aerobic chemoorganotrophic bacteria (Kelly *et al*., 2014), resulted in the presence of the pig. Like for the *S. scrofa domesticus* treatment, similar dominances were recorded by Metcalf *et al*. (2013) and Pechal *et al*. (2013). Likewise, the presence of Streptomycetaceae, aerobic Gram-positive chemoorganotrophic bacteria (Kampfer *et al*., 2014), Flavobacteriaceae, aerobic Gram-negative chemoorganotrophic non-sporulating rod-shape bacteria (McBride, 2014), Pseudomonadaceae, aerobic Gram-negative chemoorganotrophic flagellate bacteria (Brenner *et al*., 2009), Microbacteriaceae, aerobic Gram-positive bacteria (Goodfellow *et al*., 2012) and Moraxellaceae, aerobic Gram-negative chemoorganotrophic bacteria (Teixeira and Merquior 2014) were recorded for the pig.

3.7 Conclusions

This study linked observed taphonomic changes to pronounced shifts in soil bacterial community profiles as measured particularly by DGGE-derived species richness and diversity. Also, the study compared changes in microbial community structure relative to depth and used next-generation sequencing to assess the community composition in bulk control and experimental soil samples to test the programme Hypothesis 1 that the subsurface decomposition of *S. scrofa domesticus* skeletal muscle tissue as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.

Changes in the community structure relative to the burial depth showed that the bottom section recorded the highest diversity as expressed by the ecological measures possibly due to nutrient percolation in the *S. scrofa domesticus* treatment. Therefore, Hypothesis 1 was accepted when the ecological indices of richness and Shannon-Wiener and Simpson diversities were used to investigate decomposition impacts on the soil microbiome relative to burial depth.

The use of next-generation sequencing of the bulk DNA samples identified taxonomic differences in microbial community composition at order, family and species levels due to the presence of *S. scrofa domesticus* although the temporal changes were unclear with the bulk samples. The bulk analysis thus provided a scenario where a direct comparison between two sites of interest can be compared relative to depth in a real life scenario in trying to establish and understand the microbial community composition of the site of interest as an additional supportive tool in resolving crime site dispute. However further studies are needed to fully understand the complex soil microbial community of gravesites with respect to site locations.

It is suggested, therefore, that despite their known limitations, the use of molecular techniques, such as PCR-DGGE, to identify clandestine graves will be of considerable importance as an additional tool for forensic practitioners. Likewise, analysis of bulk

samples can help to resolve issues about burial site and non-burial sites. Thus, the findings of this preliminary work justified an extensive study with the introduction of an additional carbon source to establish accurately a defined timeframe for the use of PCR-DGGE profiling and temporal sequencing to determine the responses of different key microbial communities to changes in environmental and taphonomic variables that can be used in PMI estimations.

Chapter 4: Changes in soil microbiota: A decomposition case study of two carbon sources[3]

4.1 Introduction

Cadaver decomposition can be influenced by both biotic and abiotic factors, which can vary between above- and underground situations (Carter and Tibbett, 2008; Janaway *et al*., 2009). Studies which compared aboveground and underground (Campobasso *et al*., 2001; Stokes *et al*., 2009; van Belle *et al*., 2009; Carter *et al*., 2010) scenarios have shown that cadaver decomposition rate is slower in the latter. Furthermore, multiple microbial ecology techniques have been applied to: identify likely microbial taxa that are involved in and/or affected by cadaver decomposition (Allen and LaMontagne, 2015; Thomas *et al*., 2015); link microbial community composition changes with decomposition stages, *e.g.* bloat/purge and skeletonisation phases (Metcalf *et al*., 2013; Can *et al*., 2014; Hyde *et al*., 2015), and seasons (Carter *et al*., 2015); and propose use of these in PMI estimation. For example, Pechal *et al*. (2014) reported swine carcass 16S rRNA gene changes for bacterial communities at phylum and family taxonomic levels and suggested that the shifts could be used to estimate and define unique decomposition intervals. Thus, Metcalf *et al*. (2013) and Pechal *et al*. (2014) advocated the utility of the microbial community as a potential "postmortem microbial clock".

To investigate shifts in taxa communities due to the presence of decomposing carbon sources, changes in microbial community dynamics of soil only (control), *Sus scrofa*

[3] A substantive proportion of this work was published as follows:

Olakanye A.O., Thompson T.J.U. and Ralebitso-Senior T.K. 2015. Shifts in soil biodiversity - A forensic comparison between *Sus scrofa domesticus* and vegetation decomposition. *Science & Justice* **55**: 402–407.

Olakanye A.O. and Ralebitso-Senior T.K. 2018. Soil metabarcoding identifies season indicators and differentiators of pig and *Agrostis/Festuca* spp decomposition. *Forensic Science International* **288**: 53 – 58.

domesticus skeletal muscle tissue and plant litter (*Agrostis/Festuca* spp) treatments in 365-day studies were characterised and analysed by PCR-DGGE and NGS to test all three programme hypotheses [1.10]. To explore hypotheses 2 and 3, the plant litter chosen was *Agrostis/Festuca* spp) [2.3].

4.2 Experimental design

4.2.1 The study

Four g each of the *Sus scrofa domesticus* cubes and 4 g of freshly mown plant litter (*Agrostis/Festuca* spp) [2.3] were buried individually in 80 g (fresh weight) of homogenised and sieved Bishop Burton soil II [2.1] to give 1:20 (w/w) ratios. Non-supplemented soil (80 g) was used as a control. The soil was characterised [2.2] as sandy clay loam constituted (w/w) by 21% clay, 21% silt and 58% sand and physicochemical characteristics of nitrate aqueous extract as NO_3^- (76 mg l^{-1}), total organic carbon (5.9%), total S (0.05%), pH (5.8) and P (1.0 mg kg^{-1}). The study was established in triplicate sterile screw cap polyethylene containers (127 m*l*, 50 x 70 mm; VWR, Lutterworth, U.K.) for destructive sampling on days 0, 7, 14, 28, 60, 120, 180, 300 and 365 (July 2013 to July 2014) according to Table 4.1. The containers and screw caps were perforated every 7 mm (width) x 35 mm (height) for hygiene maintenance, aeration and moisture migration, filled with the fine soil fraction and maintained outdoors inside a well-perforated plastic storage boxes (Teesside University, Middlesbrough, U.K.; Lat. 54.5722° N, Long. 1.2349° W). Soil samples (5 g) were collected from multiple positions with the aid of sterile stainless steel spatula and mixed thoroughly. To prevent cross-contamination, each spatula was rinsed with 99.9% (v/v) ethanol (Thermo Fisher Scientific, Loughbrough, U.K.) after every sampling. Each sample (5 g) was stored (25 m*l* sterile universal bottles; Sarstedt, Germany) at -20°C until required for both pH measurement [2.4] and DNA extraction [2.6].

Table 4.1: Decomposition timeline by season from day 0 (July 2013) to day 365 (July 2014).

Day(s)	Month(s)	Season/Year
0 – 60	July – August	Summer 2013
120	November	Autumn 2013
180	January	Winter 2014
300	May	Spring 2014
365	July	Summer 2014

4.2.2 Data analysis

The pH, temperature, ecological indices and NGS data were analysed as described in 2.11 and 2.12.

4.3 Results

4.3.1 pH changes

Soil pH values [2.4] were measured for each of the destructively sampled triplicate controls and treatments (*S. scrofa domesticus* and plant litter) on each sampling day. The triplicate measurements were then averaged, and compared on each sampling day. pH value increases were recorded for the *S. scrofa domesticus* (6.83 ± 0.1) and plant litter (6.33 ± 0.07) treatments between days 0 and 28 compared to the control (pH 6.08 ± 0.04) (Figure 4.1) with a significant difference ($p < 0.0001$) between day 0 and 60 for the control and treatments. Decreased average pH values were recorded for the treatments between days 60 and 180 before each reached its highest value on day 300 (control, 6.44 ± 0.09; *S. scrofa domesticus,* 8.43 ± 0.01; plant litter, 7.58 ± 0.03) (Figure 4.1). Two-way ANOVA showed statistically significant temporal differences ($p < 0.001$) between the control and treatments, while the Turkey post hoc analysis identified differences ($p = 0.05$) specifically between the control and pig treatment on days 14, 28, 180 and 300.

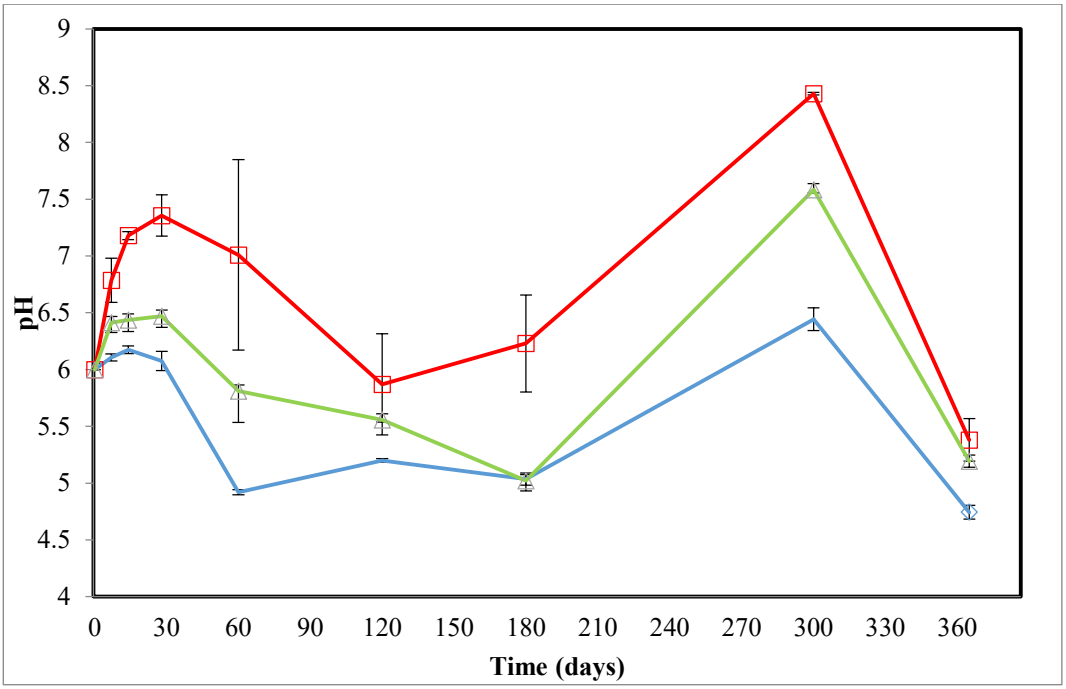


Figure 4.1: Average (n = 3) pH values of the control (◊), *S. scrofa domesticus* (□) and plant litter (Δ) treatments during 365 days of study. Bars denote standard errors.

4.3.2 Temperature

Average temperatures of the control and treatment soils were determined on each sampling day [2.5]. For accurate PMI estimation, the temperature data were further expressed in accumulated degree days (ADD), which is a model that measures the heat required for biological processes (Schoenly *et al*., 2015; Suckling *et al*., 2016). The averages of the maximum and minimum ambient temperatures were used to calculate the daily ADD (Vass *et al*., 1992; Megyesi *et al*., 2005), with a base temperature of 0°C according to earlier work of Megyesi *et al*. (2005) (Table 4.2). Between days 0 (ADD 20) and 60 (ADD 1 170.3), the average study soil temperature was 25.1°C ± 0.31 while the atmosphere was 19.2°C ± 0.19. Seasonal weather change then resulted in a temperature decrease that was marked from days 120 (ADD 1 903.7) to 180 (ADD 2 281.7) with the treatments recording an average temperature of 8°C ± 0.26. For days 300 (ADD 3 135.8) to 365 (ADD 4 012.7) study soil temperature increases were apparent with an average of 14.5°C ± 0.14 (Figure 4.2).

Table 4.2: Decomposition temperature timeline as expressed by ADD.

Day	Control	Pig	Plant litter
0	20	20	20
7	148	148.2	148.1
14	329	329.2	329.1
30	604	600.4	602.5
60	1 174.1	1 164.9	1 172
120	1 907.6	1 897.7	1 905.7
180	2 285.6	2 275.7	2 283.7
300	3 139.6	3 130.2	3 137.6
365	4 015.8	4 007.3	4 015.1

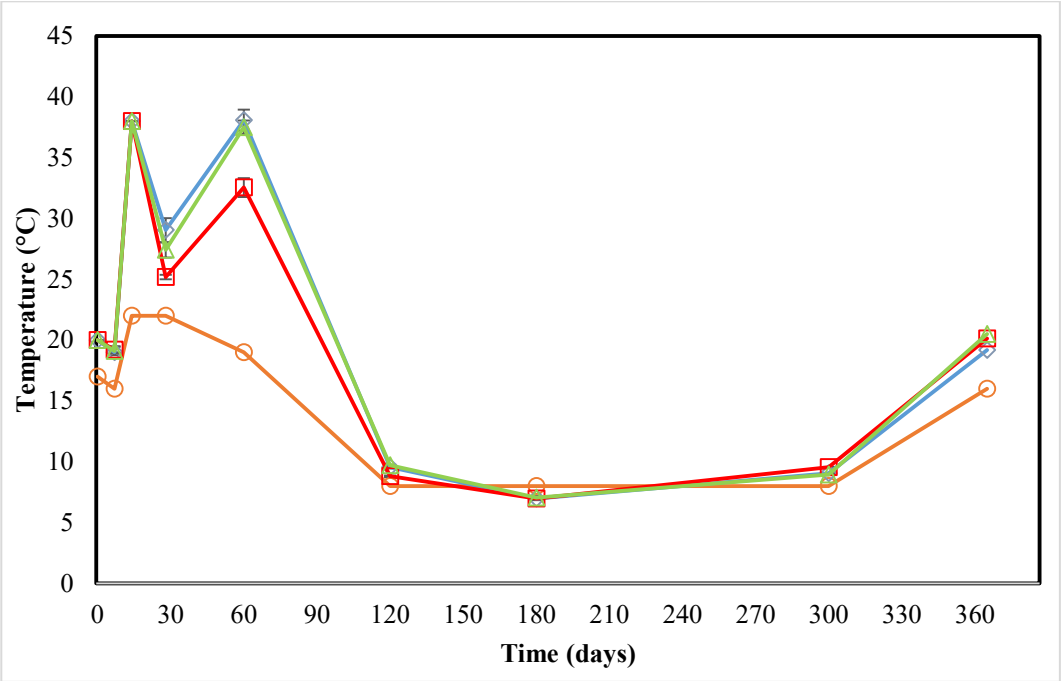


Figure 4.2: Average (n = 3) control (◊), *S. scrofa domesticus* (□) and plant litter (Δ) soil temperatures during 365 days of study. Atmospheric temperatures (○) were obtained from http://www.metoffice.gov.uk/. Bars denote standard errors.

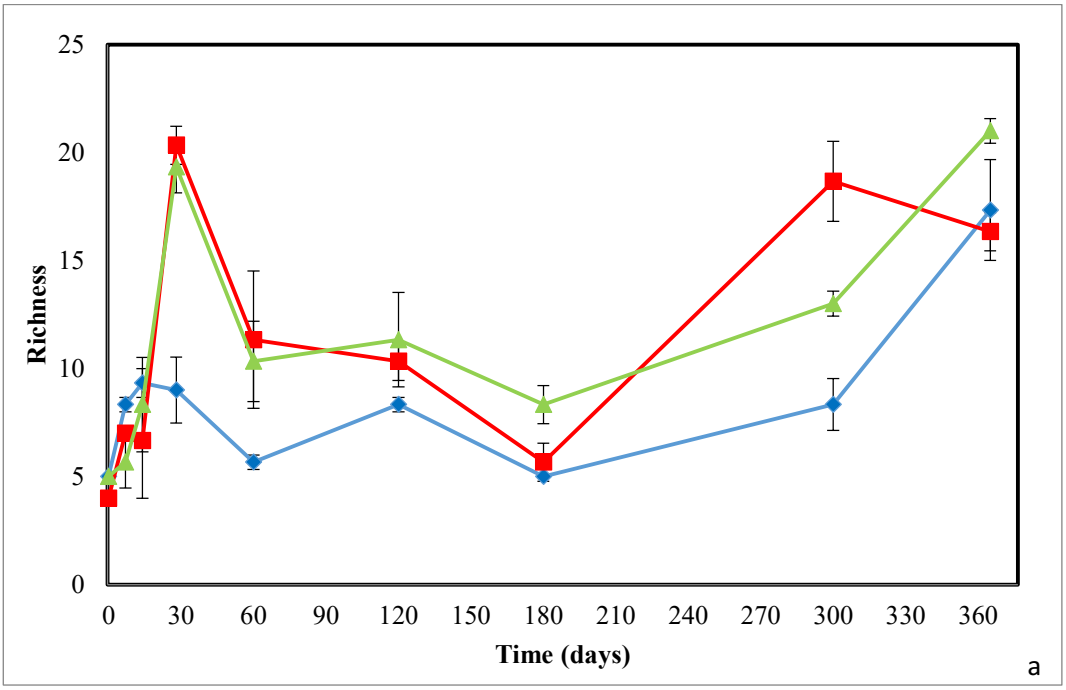
4.3.3 DGGE profile analysis

Soil ecological analysis

Richness

The DGGE fingerprints were analysed with ecological indices [2.11] to estimate the shifts in microbial communities. Species richness was calculated by the numbers of visualised DGGE bands (OTUs). Analysis of the initial 60 days (summer 2013) (Figure 4.3a) showed increases from day 0 to day 28 for the plant litter and pig treatments with a significant difference of $p = 0.024$ compared to the control. Changes were then observed with increases in richness values for the control and treatments from day 180 to day 365 with the exception of the pig treatments on day 300 where a decrease was recorded. The overall analysis of the 16S trends by two-way RMA showed statistically significant differences ($p < 0.0001$) in response to the presence of *S. scrofa domesticus* and plant litter (Figure 4.3a). Also, the Tukey (HSD) *post hoc* test identified differences ($p < 0.05$) between the control and treatments on day 28, and between the control and *S. scrofa domesticus* soil on day 300.

In contrast to the bacterial community trends, the 18S fungal profiles showed decreases from day 0 to day 60. The exceptions were the pig treatment on day 28, where an increase was observed (Figure 4.3b), and the plant litter treatment where an increase was recorded on day 60. No statistically significant differences ($p = 0.46$) resulted between the control and treatment soils within the first 60 days of study. Nevertheless, changes were observed for the plant treatments with an increase from day 28 to day 180, which was followed by a decrease from day 300 to day 365. Both the control and pig treatments recorded similar shifts with an increase between days 60 and 120, a decrease between days 120 and 180 and a final increase from day 300 to day 365. The two-way RMA analysis over the total decomposition timeline did not show any statistically significant differences ($p = 0.27$) in response to the burials. The Tukey (HSD) *post hoc* test did, however, identify differences ($p < 0.05$) between the plant litter and *S. scrofa domesticus* treatments on days 28 and 180 (Figure 4.3b).



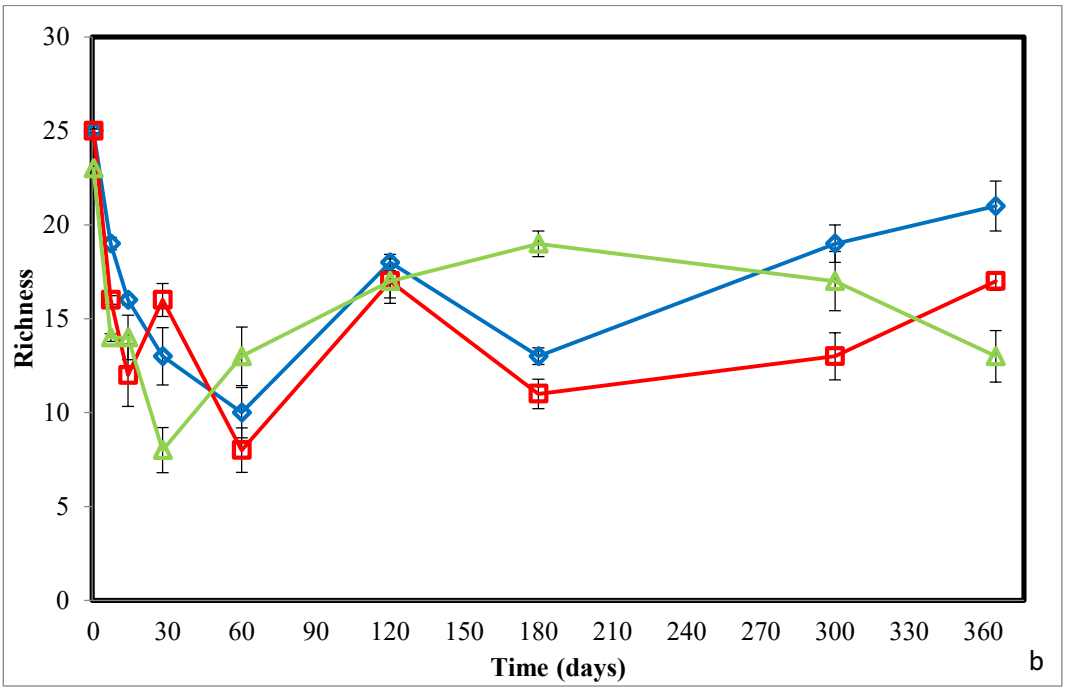
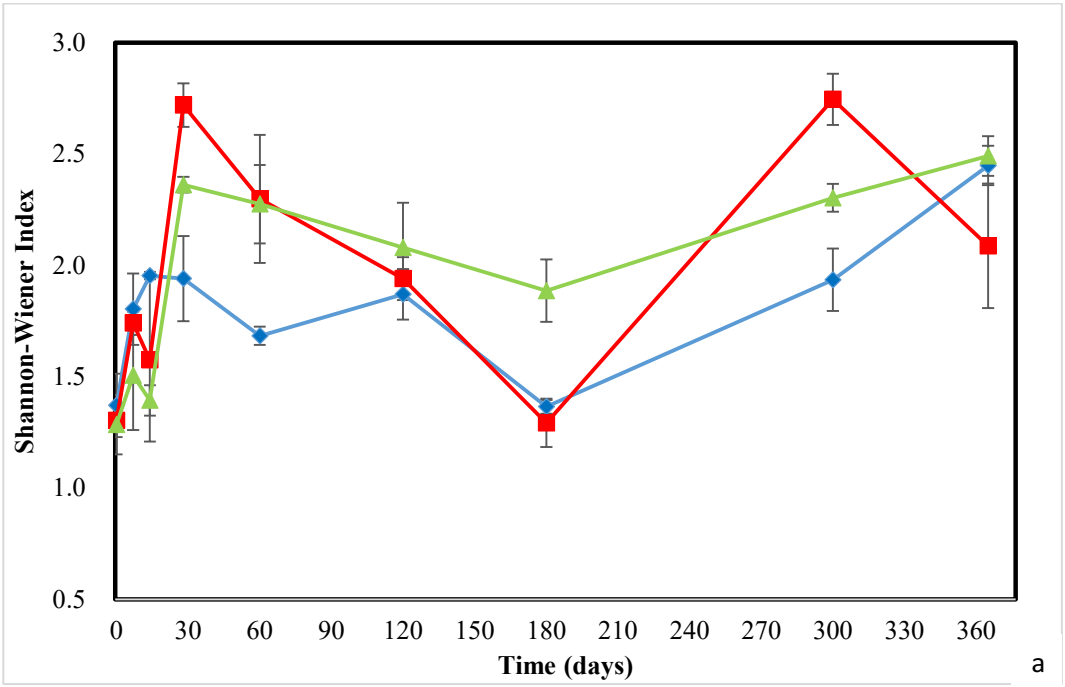


Figure 4.3: Average (n = 3) richness of 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) communities of the control (♦), *S. scrofa domesticus* (■) and plant litter (▲) treatments during 365 days of study. Bars denote standard errors.

Shannon-Wiener diversity

The 16S bacterial Shannon-Wiener index showed statistically significant temporal differences ($p = 0.034$) in diversity between the control and the *S. scrofa domesticus* and plant litter treatments. Both treatments showed a considerable increase on day 28 when compared to the control. Decreases were observed for the control and treatments from day 28 to day 180, which were followed by increases from day 180 to day 365 although the pig treatments recorded a decrease from day 300 to day 365. Final analysis with the Turkey (HSD) test identified significant differences ($p < 0.05$) between the pig treatment and control on days 28 and 300 (Figure 4.4a).

In contrast to the 16S profiles, the 18S fungal communities recorded no statistically significant differences ($p = 0.41$) between the control and experimental treatments (Figure 4.4b). Decreases were recorded for the control and treatments from day 0 to day 60 with the exceptions of the pig treatment on day 28 and plant litter treatment on day 60. While an increase was recorded for the plant litter from day 60 to day 120, this plateaued from day 120 to day 300. Both the control and pig treatment recorded similar increases between day 60 and day 120, decreases between day 120 and day 180 and final increases from day 300 to day 365. Nevertheless, the Tukey (HSD) *post hoc* test showed statistically significant differences ($p < 0.05$) between the *S. scrofa domesticus* and plant litter treatments on days 28, 60 and 180.



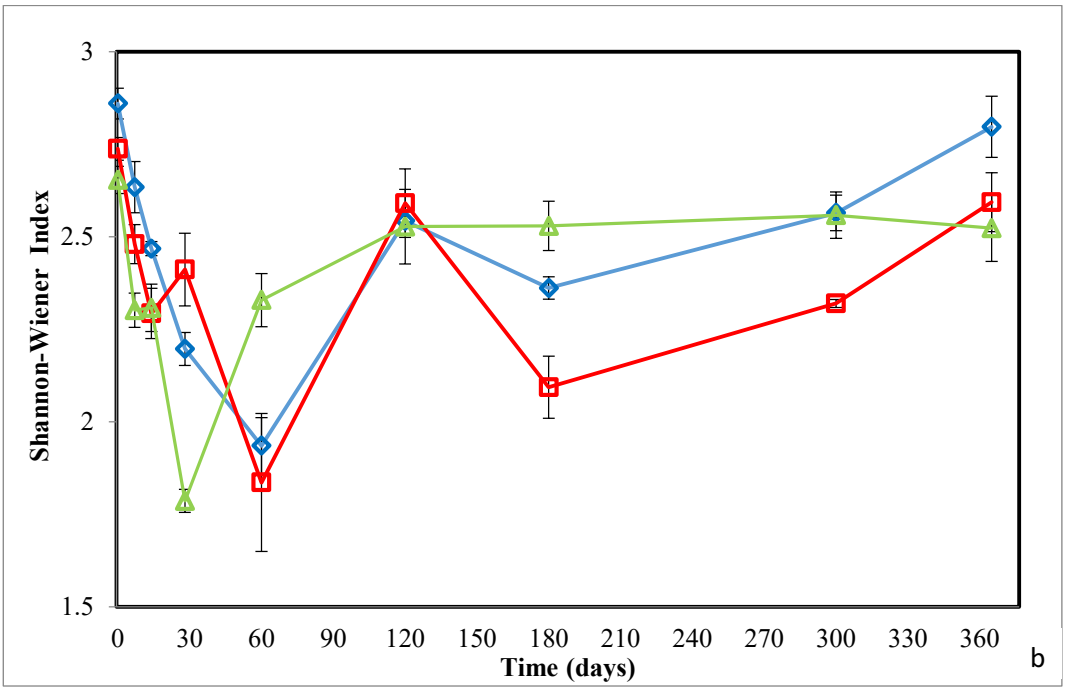
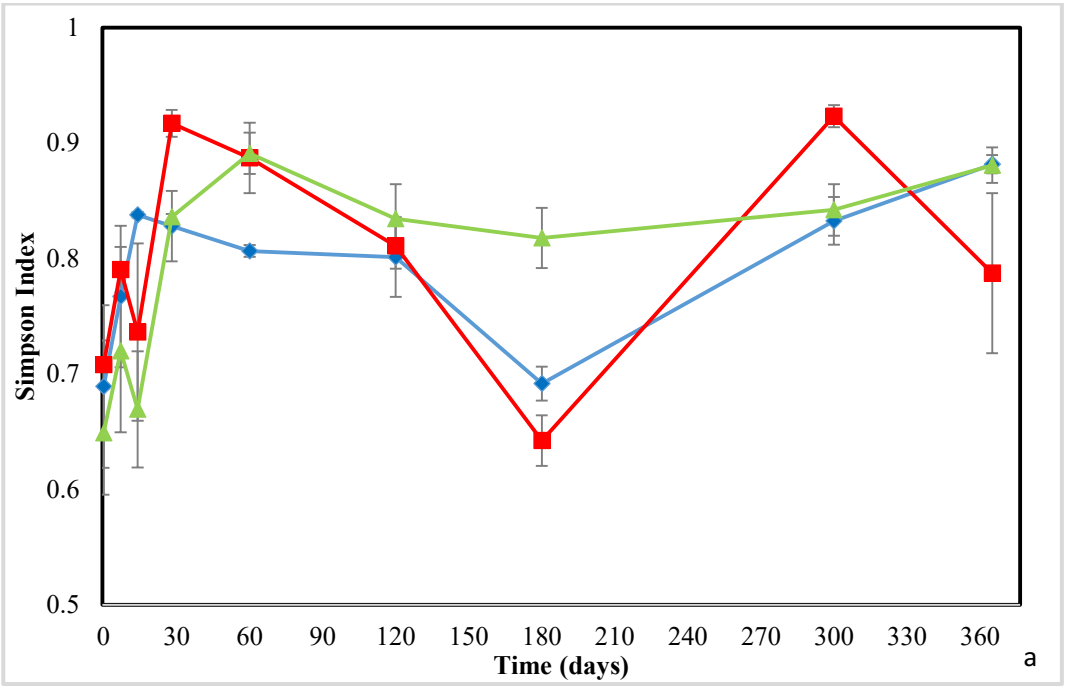


Figure 4.4: Average (n = 3) 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) communities Shannon-Wiener indices of the control (♦), *S. scrofa domesticus* (■) and plant litter (▲) treatments during 365 days of study. Bars denote standard errors.

Simpson diversity

The Simpson diversity index showed no statistically significant temporal differences (p = 0.89) for the 16S bacterial communities between the control and experimental treatments. With the exception of plant litter treatment, where an increase was observed on day 60, the control and the treatments recorded decreases from day 28 to day 180. Likewise, the control and treatments recorded increases from day 180 to day 365 while the pig treatment decreased from day 300 to day 365. Nonetheless, Tukey (HSD) *post hoc* analysis recorded differences ($p < 0.05$) on days 28 (control, 0.82 ± 0.03; *S. scrofa domesticus*, 0.92 ± 0.01), 180 (control, 0.69 ± 0.01; *S. scrofa domesticus*, 0.64 ± 0.02; plant litter, 0.82 ± 0.03) and 300 (control, 0.83 ± 0.02; *S. scrofa domesticus*, 0.92 ± 0.01) (Figure 4.5a).

For the fungal 18S rRNA gene profiles, no temporal differences were recorded (p = 0.49) for the complete decomposition timeline. With the exception of plant litter treatment on day 28, decreases were recorded for the control and treatments from day 0 to day 60. Similar changes where recorded for the control and treatments with increases between days 60 and 120, decreases between days 120 and 180 and final increases from day 300 to day 365. As seen for the bacterial communities, the Tukey (HSD) *post hoc* analysis again recorded statistically significant differences ($p < 0.05$) between the plant litter and *S. scrofa domesticus* treatments specifically on days 28, 60 and 180 and between the control and *S. scrofa domesticus* treatments on day 180 (Figure 4.5b).



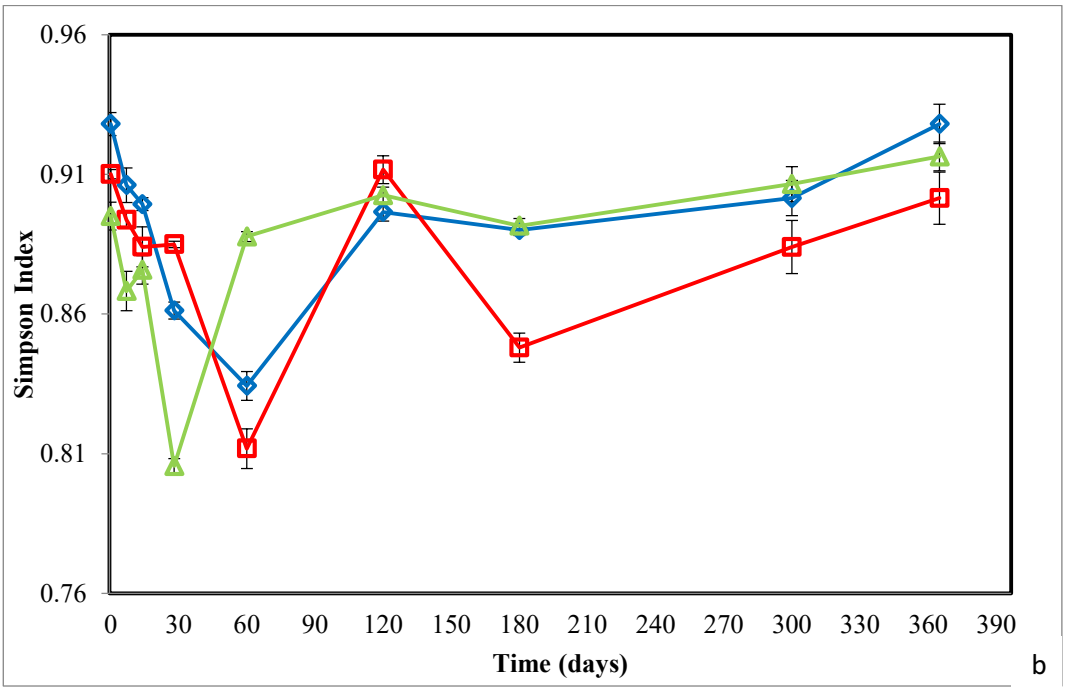


Figure 4.5: Average (n = 3) 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) community Simpson indices of the control (♦), *S. scrofa domesticus* (■) and plant litter (▲) treatments during 365 days of study. Bars denote standard errors.

Principal component analysis

The principal component analysis biplot of the ecological measures of the 16S bacterial gene (Figure 4.6a) showed that PC1 accounted for 70.15% of the variations in the clustering, while PC2 accounted for 16.80%. Generally, the early sampling times (days 7 and 14) corresponded with periods of decreased diversity, as represented on the left hand side, while the right hand side represented late (days 120 and 300) high diversity sampling intervals. Some high diversity corresponded with early sampling (day 28), which can be attributed to the randomly overlapping error bars in the richness, Shannon-Wiener and Simpson plots.

In contrast to the bacterial profiles, the 18S fungal ecological measures showed that PC1 accounted for 66.34% of the variation, while PC2 accounted for 22.45% (Figure 4.6b). The biplot showed that both the early and late sampling times recorded high diversity of the fungal community. Overall, the ecological measures of richness, Shannon-Wiener and Simpson indices for the 18S rRNA gene highly correlated throughout the study. Both the 16S bacterial and 18S fungal PC1 and PC2 scores were evaluated by ANOVA and showed no significant difference ($p < 0.05$).

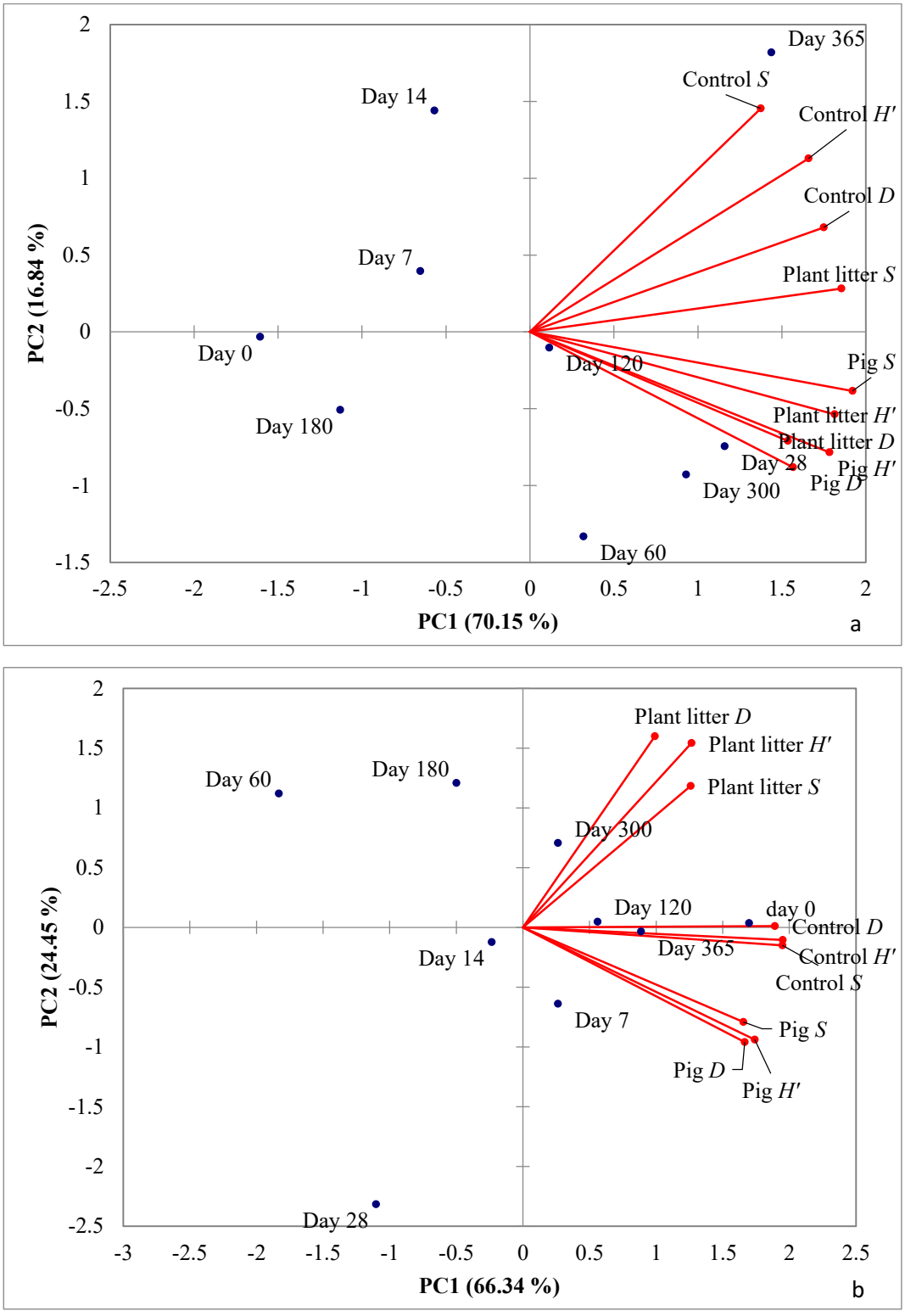


Figure 4.6: 16S bacterial (a) and 18S fungal (b) biplots for ecological measures (Shannon-Wiener, *H′*; Simpson, *D*; Richness, *S*) at specific sampling times of the control, *S. scrofa domesticus* and plant litter treatments.

4.3.4 NGS 16S Taxonomic resolution

As indicated above [4.3.3], the DGGE-based soil microbiome profiling revealed shifts in richness and diversity largely on days 28, 180 and 365, with some of the ecological indices showing mathematically significant differences on these sampling times. Therefore, they were used to investigate bulk soil trends for the community structure and composition for the 16S rRNA gene. Thus, triplicate DNA extracts from days 0, 28, 180 and 365, representing the months of July 2013, January 2014 and July 2014, were subjected to DNA purification [2.7], then pooled for the control, plant litter and *S. scrofa domesticus* treatments prior to metagenomic analysis on an Illumina platform [3.3].

Phylum-level resolution

A total of 212 248 sequences across 15 phyla (16S bacterial taxonomic resolution) were recorded for the control, *Agrostis/Festuca* spp and *S. scrofa domesticus* treatments for days 0, 28, 180 and 365. Specifically, five phyla, Actinobacteria (15.2 – 63.9%), Proteobacteria (5.8 – 54.8%), Firmicutes (3.9 – 52.5%), Bacteroidetes (0.1 – 33.3%) and Acidobacteria (0.1 – 4.7%), were dominant numerically (Figure 4.7). Only Thermodesulfobacteria (rare taxon) recorded a positive correlation with pH during the study. The Shannon-Wiener index plot (Figure 4.8) showed no statistically significant differences ($p > 0.05$) at the phylum level.

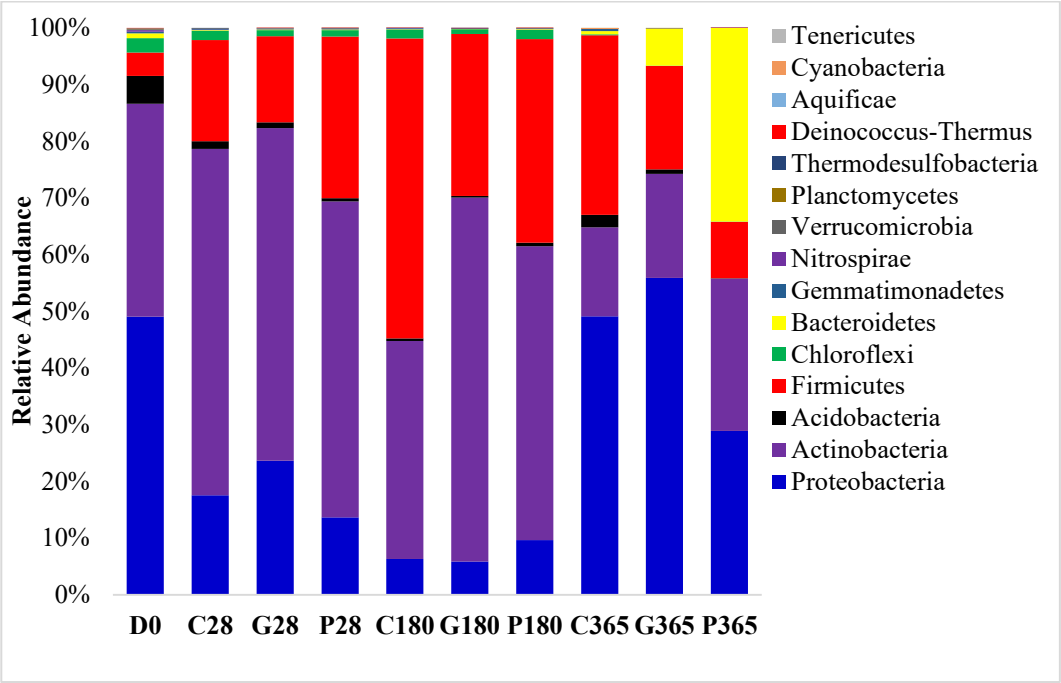


Figure 4.7: Phylum-level bacterial resolution of pooled DNA samples of the control (C), plant litter (*Agrostis/Festuca* spp, G) and pig (*S. scrofa domesticus*, P) treatments on days 0, 28, 180 and 365.

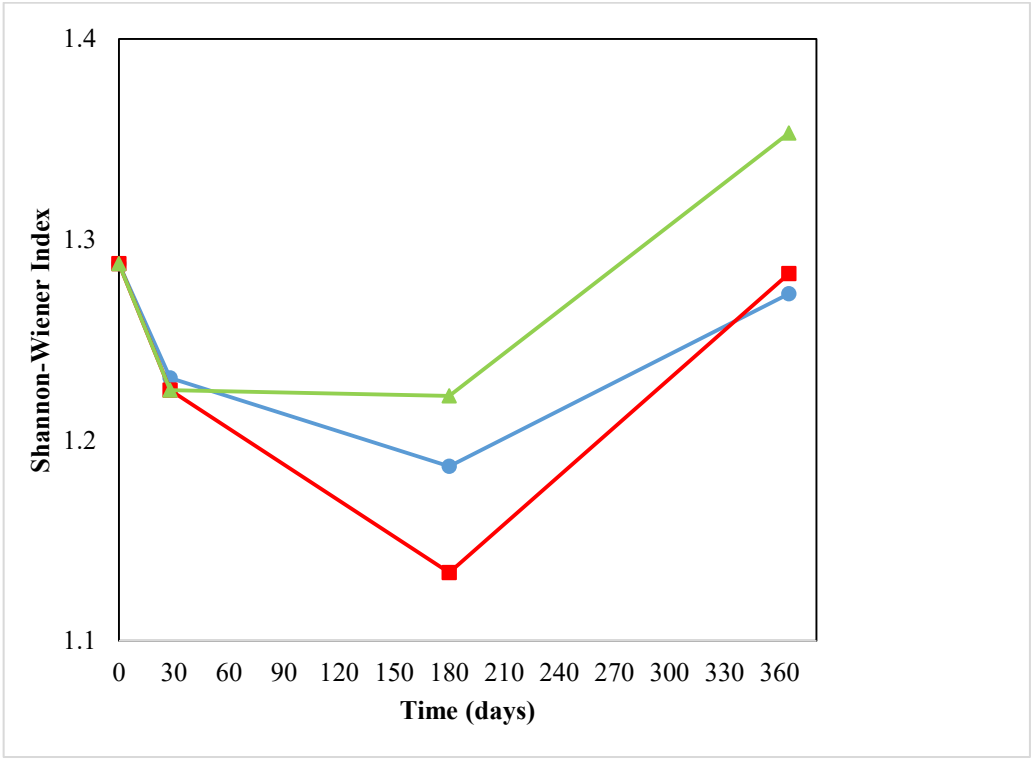


Figure 4.8: 16S bacterial taxa Shannon-Wiener index diversity plot of pooled DNA samples for the control (C ●), plant litter (G ▲) and pig (P ■) treatments on days 0, 28, 180 and 365.

Order-level resolution

Taxonomic resolution of the bacterial groups at order level revealed temporal changes in taxa relative abundances. For example, the dominant orders on day 0 were Rhizobiales and Actinomycetales (Figure 4.9). By Day 28, however, the relative abundances of Actinomycetales and Bacillales had increased while those of Rhizobiales, Xanthomonadales, Acidobacteriales, Rhodospirillales and Burkholderiales had decreased in the control and treatments. Also, for the control and pig treatment, the most dominant order on day 180 was Bacillales while for the plant litter treatment Actinomycetale dominated. Further shifts in community structure resulted by day 365 with relative abundance decreases of Actinomycetales and Bacillales for the control and treatments contrasting Rhizobiales and Burkholderiales increases. Nonetheless, different clades characterised the soils with dominances of Sphingobacteriales and Xanthomonadales recorded for the *S. scrofa domesticus* and *Agrostis/Festuca* spp treatments, respectively.

Family-level resolution

Analysis of dominant taxa at family level, including Micromonosporaceae, Rhizobiaceae, Planococcaceae, Xanthomonadaceae, Hyphomicrobiaceae and Sphingobacteriaceae, highlighted temporal and seasonal 16S community composition shifts with decomposition as revealed by heatmap analysis (Figure 4.10). For example, on day 28, Rhizobiaceae recorded differences between the control and treatments with, generally, increased relative abundance in the presence of *Agrostis/Festuca* spp. Likewise, Planococcaceae and Micromonosporaceae showed increases on day 28 for the pig treatment compared to both the control and plant litter. On day 180 (winter 2013), the pig treatment recorded Staphylococcaceae as the sole taxon shift.

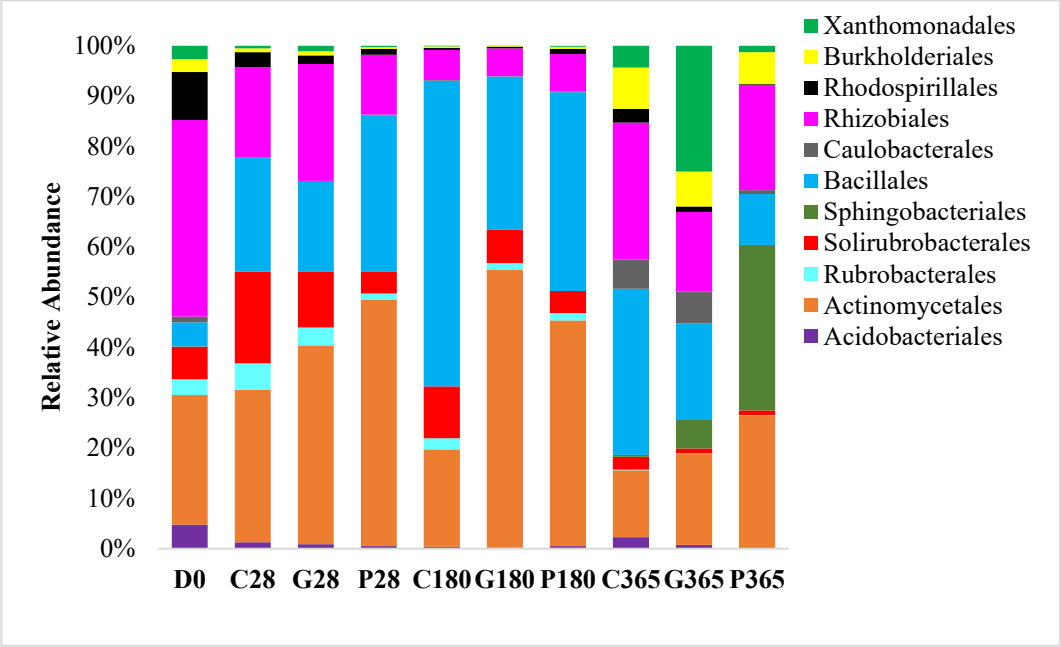


Figure 4.9: Order-level bacterial resolution of pooled DNA samples of the control (C), plant litter (*Agrostis/Festuca* spp, G) and pig (*S. scrofa domesticus*, P) treatments on days 0, 28, 180 and 365.

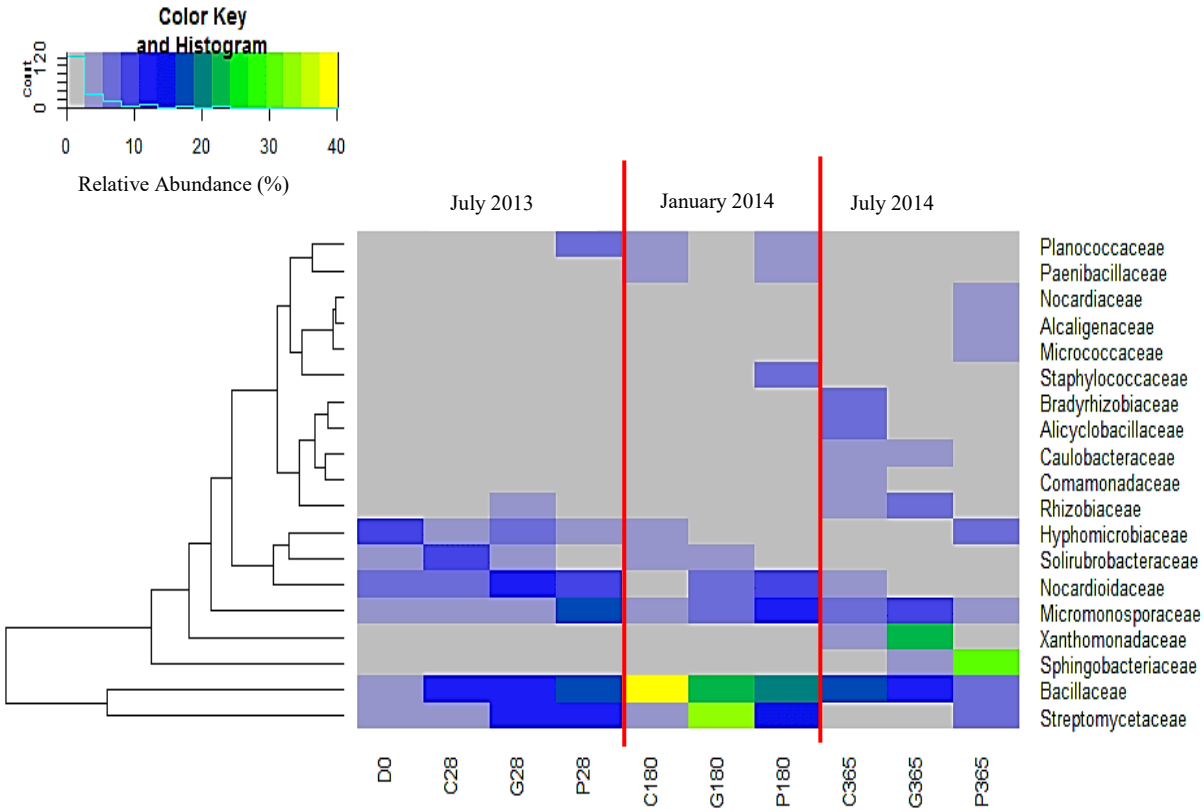


Figure 4.10: Heatmap to visualise the relative abundances (%) of the most predominant bacterial families (>0.3%) for the control (C), plant litter (*Agrostis/Festuca* spp; G) and piglet (*S. scrofa domesticus*; P) soils on days 0 (D0), 28, 180 and 365.

Unique taxa shifts such, as Alicyclobacillaceae, Xanthomonodaceae and Sphingobacteriaceae, were recorded for the control, plant litter and pig treatments, respectively, on day 365 (summer 2014). The PERMANOVA analysis between the control and treatments showed no significant difference ($p = 0.055$) although pairwise comparison with Turkey *post hoc* identified OTUs that recorded statistically significant differences at family-level resolution between the control and treatment soil samples (Table 4.3), and due to seasonal differences (Table 4.4). For example, Micrococcaceae, Caulobacteraceae and Alcaligenaceae recorded statistically significantly different abundances for the pig treatment compared to the control and plant litter soils (Table 4.3), and then between summer 2014 and the other two seasons (Table 4.4). Also, Alicyclobacillaceae recorded a statistically significant difference for the control compared to the plant litter and pig treatments ($p = 0.001$).

Table 4.3: Family-level OTUs that are statistically significantly different ($p < 0.05$) between the control and treatments soils according to the least squares means (LS-means). Combinations sharing the same letter (a, b, c) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTUs (family)	**Control**	**Plant Litter**	**Pig**	***p* < 0.05**
Micrococcaceae	0.027 b	0.036 b	1.778 a	0.000
Sphingobacteriaceae	0.112 c	1.458 b	10.034 a	0.000
Alicyclobacillaceae	2.574 a	0.360 b	0.041 b	0.001
Staphylococcaceae	0.000 b	0.017 b	2.495 a	0.000
Caulobacteraceae	1.435 a	1.767 a	0.319 b	0.002
Alcaligenaceae	0.097 b	0.324 b	1.125 a	0.003
Comamonadaceae	1.157 a	0.603 b	0.089 c	0.002
Xanthomonadaceae	1.241 b	7.688 a	0.471 c	0.000

Table 4.4**:** Family-level OTUs that are statistically significantly different ($p < 0.05$) between seasons according to the LS-means. Combinations sharing the same letter (a, b, c) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTUs (family)	**July 2013**	**January 2014**	**July 2014**	***p* < 0.05**
Micrococcaceae	0.108 b	0.115 b	1.619 a	0.000
Sphingobacteriaceae	0.014 b	0.003 b	11.588 a	0.000
Alicyclobacillaceae	0.134 b	0.144 b	2.697 a	0.001
Staphylococcaceae	0.003 c	2.317 a	0.191 b	0.000
Caulobacteraceae	0.246 b	0.043 b	3.232 a	0.002
Alcaligenaceae	0.161 b	0.040 b	1.346 a	0.003
Comamonadaceae	0.125 b	0.015 b	1.709 a	0.002
Xanthomonadaceae	0.289 b	0.018 b	9.093 a	0.000

Genus-level resolution

The two-dimensional NMDS (stress = 0.15) of the bacterial community structure at genus level identified temporal differences with days 28 (summer 2013) and 180 (winter 2014), which were significantly different from day 365 (summer 2014) (Figure 4.11). For example, *Hyphomicrobium* and *Solirubrobacter* decreased from 6.78% and 2.89% on day 0 to <1% and below detection on day 365, respectively (Figure 4.12). Also, *Kribella* was detected (5.45% – 10.84%) until day 180 but not on day 365. In contrast, dominances of *Rhodanobacter* (18.12%) and *Dyella* (3.04%) were recorded only on day 365 for the *Agrostis/Festuca* spp treatment while increases in *Pedobacter* and *Devosia* were recorded at the same sampling time from the pig treatment.

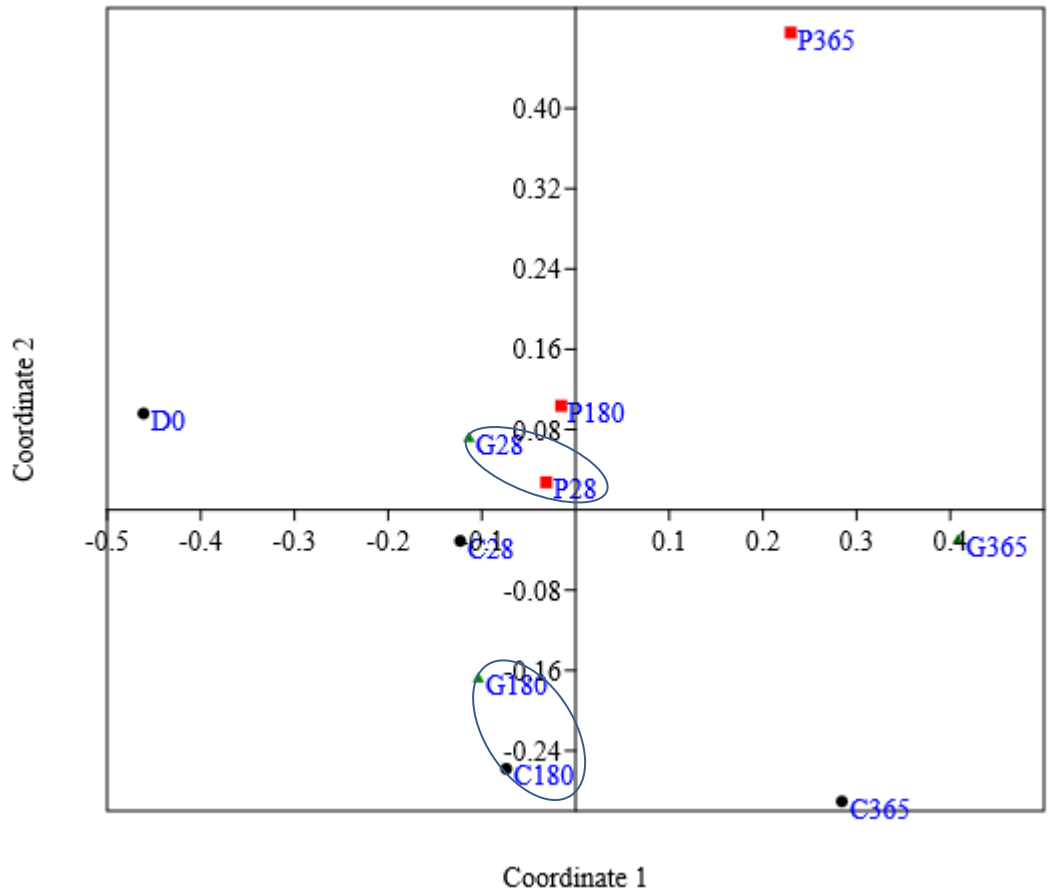


Figure 4.11: NMDS (stress = 0.15) plot for 16S bacteria community at genus level for the control (C; ●), plant litter (G; ▲) and pig (P; ■) treatments on days 0, 28, 180 and 365.

Other taxonomic shifts included increases in the relative abundances of *Shinella* (4.63%) and *Micromonospora* (16.27%) on day 28 for the plant litter and *S. scrofa domesticus* treatments, respectively. Together with overall community structure differences with time, taxa abundances were examined further to identify treatment-specific indicators, particularly on day 365. The *S. scrofa domesticus* treatment recorded dominances of *Pedobacter* (24.14%) and *Devosia* (6.31%) compared to the plant litter (1.31%; 1.78%) and control (0.01%; 0.15%) (Figure 4.12). Specifically, *Sphingobacterium* was absent in the plant litter treatment but recorded a 5.92% dominance in the *S. scrofa domesticus* treatment. Similarly, *Arthrobacter* and *Rhodococcus* were not recorded for the control but were present at 4.85% and 3.53% abundances, respectively, in the pig treatment.

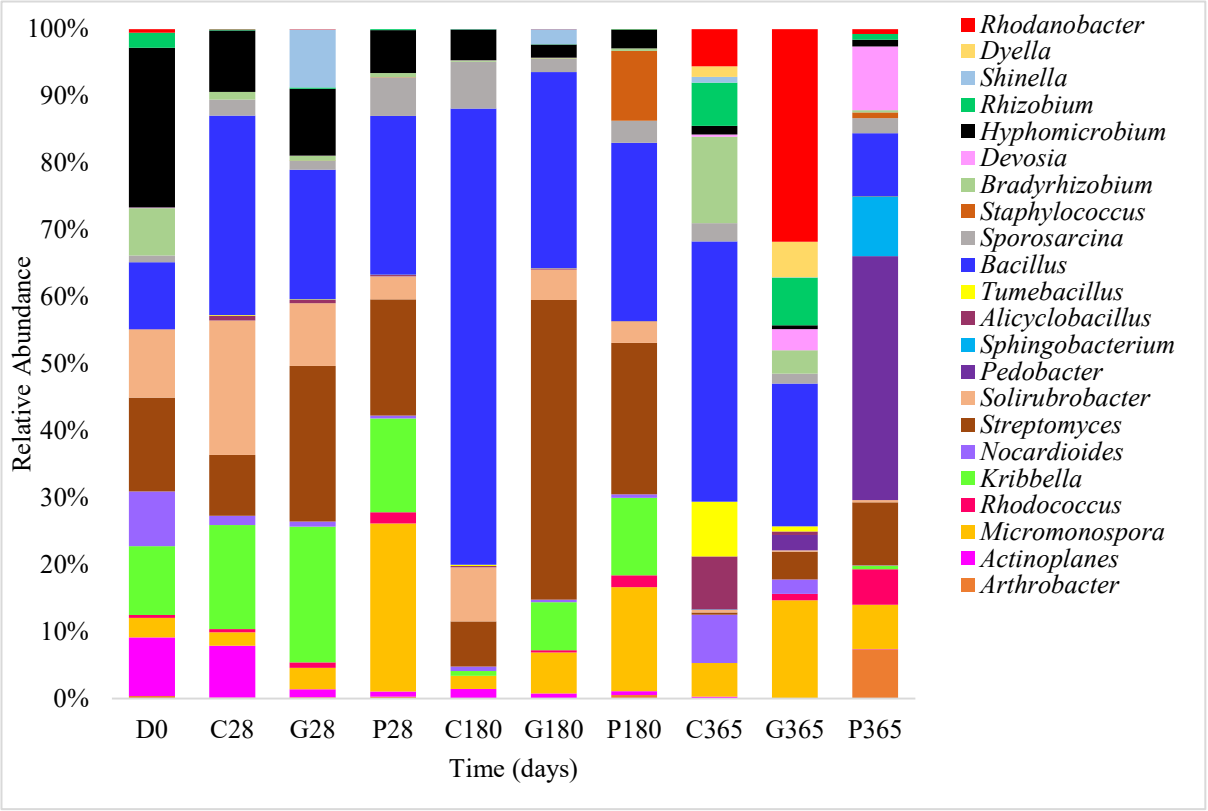


Figure 4.12: Bacterial taxa (genus) resolution of pooled DNA samples for the control (C), plant litter (*Agrostis/Festuca* spp, G) and pig (*Sus scrofa domesticus*, P) treatments of days 0, 28, 180 and 365.

4.4 Discussion

Studies of cadaver decomposition and its interactions with, and effects on, soil ecology have highlighted the potential of forensic ecogenomics as a powerful tool to estimate PMI and identify clandestine graves through changes in microbial communities (Hyde *et al*., 2013; Lauber *et al*., 2014). Although this tool has potential advantages compared with conventional methods for estimating PMI, most studies have, however, only considered a single carbon source (the cadaver) while dual sources can provide useful information for forensic practitioners to identify and differentiate gravesites in difficult cases such as transit or clandestine scenarios. This study, therefore, examined the effects of two carbon sources, *S. scrofa domesticus* and plant litter (*Agrostis*/*Festuca* spp) on soil microbiome dynamics, with the latter intended to mimic domestic crime scenarios where garden grass litter is incorporated into clandestine burials.

pH and temperature

Changes in soil pH due to cadaver decomposition have been reported (Reed 1958; Vass *et al*., 1992; Hopkins *et al*., 2000) and Metcalf *et al*. (2016) attributed these to ruptured cadaver spillage of ammonia-rich fluid. The results of the current study showed differences in pH in response to *S. scrofa domesticus* and plant litter decomposition most probably due to their chemical compositions of protein, lipid and carbohydrate (Forbes, 2008; Janaway *et al*., 2009) and cellulose and lignin (Moretto *et al*., 2001; Tagliavini *et al*., 2007), respectively. In general, Hopkins *et al*. (2000) reported an accumulation of NH_4^+ and mineralisation of C and N at a gravesite, which resulted in a soil pH increase similar to the one recorded for the *S. scrofa domesticus* treatment. Thus, although NH_4^+, PO_4^-, K^+ and NO_3^- were not analysed in the current study, the recorded pH changes can still be related to earlier research (Hopkins *et al*., 2000; Forbes, 2008) which entailed physicochemical characterization of gravesoil. Consequently, changes in pH can result in microbial population shifts with bacteria more dorminant in pH 5.5 – 7.5 gravesoil (Finley *et al*., 2015a).

Ambient temperature has been shown to have one of the greatest effects on cadaver decomposition rate. According to various workers (*e.g.* Tibbett *et al.,* 2004; Carter *et al*., 2008; Stokes *et al*., 2009), the rate is enhanced by high temperature while the process slows or stops at cold temperatures. For cadaver decomposition under anaerobic conditions the optimum temperature range is 21 to 38°C, with very limited activity at temperatures below 4°C (Mann *et al*., 1990; Janaway *et al*., 2009; Zhou and Byard, 2011). Although not controlled as illustrated in a seminal laboratory-based investigation by Tibbett *et al*. (2004), increases and decreases in treatment soils temperatures in the current outdoors investigation were linked to seasonal shifts were and accompanied by increases and decreases in diversity of both the 16S bacterial and 18S fungal microbiome communities.

DGGE-based ecological indices

The use of some ecological indices, as employed by other researchers (Bandeira *et al*., 2013; Barry *et al*., 2013) revealed interesting data that could be used to differentiate between *S. scrofa domesticus* and plant litter decompositions. As suggested by McGuire and Treseder (2010), bacterial and fungal richness might be useful in calculating decomposition time. Likewise, Parkinson *et al*. (2009) highlighted the potentials of soil bacterial and fungal communities as time-since-death biomarkers. For this study, ecological indices determined for 16S rRNA bacteria communities seemed to be the most useful for estimating PMI for *S. scrofa domesticus*. For example, bacterial diversity and richness indices highlighted some temporal differences particularly on days 28 (summer; July 2013), 180 (winter; January 2014) and 300 (summer; July 2014) (Figures 4.4a, 4.5a and 4.6a). In contrast, 18S rRNA fungal determinations were the best estimates of plant litter decomposition (Figures 4.4b, 4.5b and 4.6b). As reported by Voriskova and Baldrian (2013), fungi tend to dominate the later stage of plant decomposition and this was evidenced by the plant treatment diversity measurements (Figures 4.4b, 4.5b and 4.6b).

The elevated temperature on day 14 led, potentially, to marked increases in diversity of the 16S bacterial community as expressed by ecological measures for the *S. scrofa domesticus* and plant litter treatments, while the 18S fungal community recorded an increase for the *S. scrofa domesticus* treatment only. In contrast, the drop in temperature from an average of 32°C (day 60) to around 8°C as observed from day 120 to day 180 resulted in a decrease in diversity of the 16S bacterial community for the *S. scrofa domesticus* and an increase in the 18S fungal community for the plant litter as expressed by ecological measures of richness, Shannon-Wiener and Simpson diversity indices.

Overall, principal component analysis showed differences recorded between the 16S bacterial and 18S fungal communities depending on the ecological index measured. For example, an increase in 16S bacterial diversity from day 0 to day 28, as expressed by

ecological measures of richness (Figure 4.4a), Shannon-Wiener index (Figure 4.5a) and Simpson index (Figure 4.6a), for *S. scrofa domesticus* and plant litter, contrasted the 18S fungal communities (Figures 4.4b, 4.5b and 4.6b). In general, this analytical tool, which is recommended to ensure the robustness of results from multivariate studies, also proved useful to test and validate the changes recorded from multiple parameters and DGGE-derived data of this decomposition study.

Microbial community taxonomic resolution

To better understand key temporal interactions between soil ecology and cadaver decomposition, microbial community dynamics were compared in the presence of two different carbon sources: *S. scrofa domesticus*, as a human taphonomic proxy; and *Agrostis/Festuca* spp, as a non-pig organic material. On day 0, Proteobacteria accounted for ≥ 50% of the total community with *Hyphomicrobium*, a genus consisting of chemoorganotrophic rod-shaped denitrifying bacteria (Mills *et al*., 2008), belonging to Alphaproteobacteria, dominant. Taxonomic community shifts then resulted with decreases in Proteobacteria and increases in Actinobacteria and Firmicutes as reported previously (Janaway *et al*., 2009; Pechal *et al*., 2014; Hyde *et al*., 2015), but on day 28 of this study. Characteristically, these taxa have been associated with soil microbiota, the human microbiome and meat spoilage (Pechal *et al*., 2014).

Overall, the 16S community structure varied in the presence of pig and plant litter. Family-level comparisons recorded temporal divergences between the control and treatment soils, with the differences considerably pronounced on day 365 (Figure 4.10). Specifically, ubiquitous families such as Micromonosporaceae and Bacillaceae were recorded for the entire decomposition timeline but with the former, in particular, recording highly consistent relative abundances throughout the trial. The taxon would, therefore, be an identifier for this soil type but neither a target for pig/plant litter decomposition nor a microbial clock indicator. In contrast, Staphylococcaceae was recorded solely for the *S. scrofa domesticus*

treatment after 180 days with no specific differentiators for the control and *Agrostis/Festuca* spp soils, making this family a decomposition indicator for the human analogue and winter season. Further to this, new taxa that were often exclusive of one treatment or the control were recorded after 365 days. For example, Norcardioidaceae, Comamonadaceae, Alicyclobacillaceae and Bradyrhizobiaceae characterised the control while Nocardiaceae, Alcaligenaceae, Micrococcaceae and Hyphomicrobiaceae were unique to the pig soils. Finally, Xanthomonadaceae and Sphingobacteriaceae were the only families that recorded increased numerical abundances after 365 days that were unique to the plant litter and pig soils, respectively. Thus repeat studies on this soil type, using the same decomposition substrates and timeline, could target the temporally unique families towards understanding the dynamic processes within the soil microbiome community.

Group comparisons revealed the predominance of the aerobic Gram-positive bacteria *Micromonospora* and *Sporosarcina* (De-Vos *et al*., 2009; Goodfellow *et al*., 2012) for the *S. scrofa domesticus* treatment and the occurrence of *Shinella*, aerobic Gram-negative nitrogen-fixing symbiotic bacteria (Perry and Yost, 2014), for the plant litter treatment on day 28. While Cobaugh *et al*. (2015) observed the presence of *Shinella* in the advanced stage of human cadaver decomposition, its dominance was observed in the current study for the plant litter treatment only. This contrasted the dominance of *Actinoplanes*, Gram-positive, spore-forming mycelium aerobic bacteria (Goodfellow *et al*., 2012), and *Solirubrobacter*, Gram-positive aerobic bacteria (Goodfellow *et al*., 2012) for the control soil. The dominance of *Solirubrobacter* on day 28 (summer 2013) for the control contrasted the work of Carter *et al*. (2015) who reported increased abundance of Solirubrobacterales in the winter period. In addition, a seasonal shift in the current study from summer to winter between days 28 and 180 resulted in the dominance of *Bacillus* for the control and treatment soils. The presence of *Staphylococcus*, Gram-positive facultative anaerobic bacteria (De-Vos *et al*., 2009), for the *S. scrofa domesticus* treatments on day 180 identified a potentially useful community temporal and seasonal indicator for winter in this soil type.

The subsequent seasonal change from winter to summer resulted in a dominance of Proteobacteria on day 365 for both the control and plant litter, which contrasted a dominance of Bacteriodetes in the *S. scrofa domesticus* treatment. Also, temporal changes resulted in the predominance of Firmicutes in the control soil. Variations at the genus level characterised the control and treatments with the presence of *Alicyclobacillus* and *Tumebacillus*, aerobic Gram-positive chemoorganotrophic bacteria from the family Alicyclobacillaceae (De-Vos *et al*., 2009), and chemolithoautotophic *Bradyrhizobium* (Pearce *et al*., 2014) for the control and *Rhodanobacter* for the *Agrostis/Festuca* spp treatment. Also, increases in *Rhizobium*, Gram-negative symbiotic bacteria of the phylum Proteobacteria involved in nitrogen fixation, deamination, ammonification and denitrification (Perry and Yost, 2014), were recorded for the control and plant litter. Likewise, the pig treatment was characterised by *Rhodococcus*, *Arthrobacter*, *Pedobacter, Devosia* and *Sphingobacterium*, aerobic Gram-negative bacteria from the family Sphingobacteriaceae that contain sphingophospholipid and ceramides in their cell membranes (Krieg *et al*., 2010). Increases in the relative abundance of Sphingobacteriaceae (Bacteroidetes phylum) have been reported previously during decomposition (Metcalf *et al*., 2013; Carter *et al*., 2015) as was observed in the pig treatment particularly during summer 2014.

Taxa analyses, which emphasised dominance rather than presence/absence, suggested that Micromonosporaceae/*Micromonospora* were likely early (day 28) PMI indicators of pig decomposition at family/genus level. *Devosia,* Sphingobacteriaceae/*Sphingobacterium*, *Pedobacter* and Xanthomonadaceae/*Rhodanobacter* were then seasonal (summer) PMI markers that also differentiated between pig and plant litter during late (day 365) decomposition. Furthermore, while Metcalf *et al*. (2013) associated a predominance of the Xanthomonadaceae with mouse decomposition, their decrease in the *S. scrofa domesticus* treatment aligned the current findings to the work of Hyde *et al*. (2015) and Pechal *et al*. (2014) who used human cadaver and swine carcass. In particular, the heatmap identified

Sphingobacteriaceae as a key family indicator for summer at 365 days since interment. Also, *Arthrobacter* and *Rhodococcus* were ideal microbial clocks to differentiate between control and pig decomposition in the sandy clay loam soil.

Overall analyses of the decomposition timeline identified Micrococcaceae, Gram-positive aerobic bacteria; Sphingobacteriaceae; Staphylococcaceae, Gram-positive facultative anaerobic bacteria (De-Vos *et al*., 2009); and Alcaligenaceae, aerobic Gram-negative rod or coccobacilli chemoorganotrophic bacteria (Whiteson *et al*., 2014), which were associated with advanced stages of decomposition (Metcalf *et al*., 2013), as statistically significantly different ($p < 0.05$) for the pig interment compared to both the control soil and *Agrostis/Festuca* spp litter treatment. While Alicyclobacillaceae, aerobic Gram-positive chemoorganotrophic bacteria, and Comamonadaceae, aerobic Gram-negative bacteria commonly found in soil and water habitats (Willens, 2014), were significantly different ($p < 0.05$) for the control soil, only Xanthomonadaceae, aerobic Gram-negative straight rod obligate bacteria (Brenner *et al*., 2009), were significantly different ($p < 0.05$) for the plant litter treatment. Likewise, seasonal taxa differences were recorded mostly during summer 2014 with taxa such as Micrococcaceae, Sphingobacteriaceae, Alicyclobacillaceae, Comamonadaceae, Xanthomonadaceae and Alcaligenaceae significantly different ($p < 0.05$) when compared to both summer and autumn 2013. Only Staphylococcaceae were significantly different ($p < 0.05$) during winter 2013.

4.5 Conclusions

Abiotic factors such as temperature and pH are key variables in taphonomic studies. Therefore, they can be manipulated to assess their respective impacts on decomposition or, conversely, monitored as indications of the impacts of decomposition on the surrounding ecosystem. For this study, the latter approach was applied where soil pH and temperature were measured at every sampling time. Significant differences were observed between the control and treatments, with the pig soil pH readings recording the highest values. These

differences can be attributed to various organic metabolic by-products from the carbon/nitrogen sources.

This study used destructive sampling with 80 g soil to test the three programme hypotheses [1.1]. Overall, the research identified interesting trends, as revealed by DGGE as an accessible ecogenomics technique, and robust/multiple statistical analyses, which could be useful in identifying clandestine graves by targeting specific microbial community diversity changes. Specifically, ecological indices determined for 16S rRNA bacteria communities seemed to be the most useful for estimating PMI for *S. scrofa domesticus* while 18S rRNA fungal ecological indices were the better indicators of plant litter decomposition. Therefore, Hypotheses 1 and 2 were accepted when using ecological indices of richness, Shannon-Wiener and Simpson diversity, but relative to specific microbial groups.

Likewise, the next-generation sequencing of pooled DNA samples for the forensic ecogenomic analysis identified taxonomic changes at both family and genus levels due to the presence of decomposing material, particularly *S. scrofa domesticus*, with temporal effects recorded for the bacterial communities. Therefore, Hypotheses 1 and 3 were accepted with the use of next-generation sequencing. The results further suggested that non-burial soils and gravesoils can be differentiated at both family and genus level with the possibility of a bacterial clock to estimate postmortem interval/time-since-burial.

The bacterial taxon *Bacillus* dominated the soil controls in comparison with the *S. scrofa domesticus* burial and so excluded this genus as a microbial clock indicator for this soil type. Notwithstanding this, shifts in clades such as *Sphingobacterium* and *Rhodanobacter* provided preliminary evidence for 16S-based divergences between pig and plant litter decomposition. In particular, the sole incidence of *Sphingobacterium* and the marked dominance increase of *Pedobacter* in the presence of the mammalian taphonomic proxy, concomitant with the absence of the former in the control and plant litter treatment, identified them as (seasonal) microbial clock indicators for the sandy clay loam soil. Similarly, some

families were recorded exclusively for the control, pig and plant litter at the end of the study (day 365). These families/genera could, therefore, be potentially targeted to identify/and or predict the presence of a body, 365 days after interment.

This is the first research where microbial decomposer communities of two carbon sources were compared in a forensic context to further knowledge of microbiomes. The study reflects next-generation sequencing analysis where pooled DNA samples provided a preliminary investigation of bulk microbial taxonomic differentiation temporally between the presence and absence of a decomposing human cadaver analogue, and in comparison to plant litter. The approach reflected real cases more closely where soil microbial community analyses from trace evidence, often with no opportunities for replicates, would parallel other forensic intelligence gathering for suspected clandestine grave locations. While this study is novel and provided further insight of the soil microbiome community, it was made with processed soils for maxima of 365 days. Therefore, future *in situ* investigations should be made with: (i) different unprocessed soil types; (ii) whole cadavers/mammalian proxies; (iii) various plant litters; (iv) different start dates within the same year; and (v) same start dates/seasons across different years, with relevant attendant mathematical/statistical analyses for comprehensive analyses of subsurface microbiomes.

Chapter 5: A preliminary decomposition study with whole piglet, plant litter and a mixture of both[4]

5.1 Introduction

A cadaver is an energy resource, which plays a role in nutrient cycling with the release of numerous compounds, such as acetic acid, amino acids and propionic acid, into the surrounding soil (Cobaugh *et al*., 2015; Metcalf *et al*., 2016). In particular, its decomposition is often described as a complex process that is attributed to microbial, vertebrate and invertebrate scavenger metabolic activities, which impact the surrounding environmental microbiota (Pechal and Benbow, 2015; Metcalf *et al*., 2016). Advancements in molecular microbial ecology techniques have enabled researchers to study the epinecrotic and necrobiome communities in these complex interactions with cadaver decomposition. As an extension of the work discussed in Chapter 4, where 4 g of two carbon/energy sources (*Sus scrofa domesticus* and *Agrostis/Festuca* spp) recorded differences in their decomposition-impacted soil microbial communities, this study was made with whole stillborn piglets (*S. scrofa domesticus),* plant litter (*Agrostis/Festuca* spp) and a mixture of both to address the overarching research hypotheses [1.10]: (1) whole *S. scrofa domesticus* carcass subsurface decomposition would change the structure and composition of the surrounding soil microbiome; (2) whole *S. scrofa domesticus* carcass subsurface decomposition would effect different shifts in the surrounding soil microbiome structure and composition when compared to plant/leaf litter; and (3) seasonal variations would influence shifts in soil

[4] A substantive proportion of this work was published as follows:

Olakanye A.O. and Ralebitso-Senior T.K. 2017. Assessing subsurface decomposition and potential impacts on forensic investigations. In: Ralebitso-Senior TK (ed.). *Forensic EcoGenomics: The Application of Microbial Ecology Analyses in Forensic Contexts* (pp 145 – 176). London: Academic Press.

microbiome structure and composition during *S. scrofa domesticus* and plant/leaf litter decompositions as expressed by 16S taxa distributions.

5.2 Experimental design

5.2.1 The study

To explore further the trends for 16S bacterial and 18S fungal genes observed in Chapter 4 for *S. scrofa domesticus* cubes and plant litter (*Agrostis/Festuca* spp), a more expansive study was made with whole stillborn piglets, plant litter and a mixture of both (Figure 5.1). Thus, frozen (-20°C) stillborn piglets (~ 1.5 kg) were sourced from Northumbria Police (Ponteland, U.K.), transported on icepacks, re-frozen (-20°C) and thawed completely and immediately before the study burials. Soil III from a well secured site at Framwellgate Moor, County Durham, U.K. (Lat. 53.15°N, Long. 1.59°W) was homogenised and sieved [2.1]. The soil was characterised [2.2] as sandy clay loam constituted by (w/w) 26% clay, 21% silt and 53% sand and physicochemical characteristics of nitrate aqueous extract as NO_3^- (4.6 mg l^{-1}), total organic carbon (4.1%), total S (0.03%), pH (6.3) and P (1.2 mg kg^{-1}).

Four sterile 30 *l* polyethylene storage boxes (46.5 x 36 x 25.5 cm; Asda, U.K.) were used and the study established as follows: 20 kg soil (control); piglet (1.64 kg) buried in soil (32.8 kg); plant litter (1.5 kg) buried in soil (30 kg); and piglet (1.32 kg) plus plant litter (1.2 kg) buried in soil (50.4 kg) in pariy with the earlier studies (Chapters 3 and 4) where the carbon sources to soils ratio were 1:20 (w/w).

Each box was perforated at equal distances and heights (6 cm) on the sides, bottom and lid to facilitate aeration, moisture migration, sampling and hygiene maintenance. The boxes were maintained outdoors (Teesside University, Middlesbrough, U.K.) at ambient temperature elevated on bricks within plastic trays for hygiene maintenance.

Soil samples were collected from multiple positions with the aid of sterile stainless steel spatulas for the first two weeks then monthly for 10 months from November 2014 to

September 2015 (Table 5.1). To prevent cross-contamination, the spatula was rinsed with 99.9 % (v/v) ethanol (Thermo Fisher Scientific, Loughbrough, U.K.) between samplings. Composites of the homogenised (10 g) samples were stored (25 m*l* sterile universal bottles; Sarstedt, Germany) at -20°C until required for both pH measurement [2.4] and DNA extraction [2.6].

Table 5.1: Decomposition timeline by season from day 0 (November 2014) to day 365 (September 2015).

Day(s)	**Month(s)**	**Season/Year**
0 – 14	November	Autumn 2014
30 – 90	December – February	Winter 2014
120 – 180	March – May	Spring 2015
210 – 270	June – August	Summer 2015
300	September	Autumn 2015

5.2.2 Next-generation sequencing

The purified microbial community DNA extracts [2.7] were sequenced with an Illumina Miseq platform (NU-OMICS, Northumbria University, Newcastle Upon Tyne, U.K.) with a primer set targeting the V4 region of the bacterial 16S rRNA gene as described previously by Kozich *et al*. (2013). The raw sequencing reads were processed in FASTQ format and were analysed with Mothur software package (version 1.36.1) (University of Michigan, U.S.A.). The FASTA formatted sequences were quality checked and filtered with UCHIME. The sequences were aligned to the SILVA reference and taxonomic identification of the reads were assessed by assigning sequences to OTUs with Ribosomal Database Project (RDP) classifier. PCR negative controls were run and sequenced in parallel to the samples with OTUs present in negative controls and samples excluded from further analysis. Non-bacterial sequences (*e.g.* archaea) were discarded and reads rarified at 14 328 sequences per

sample. OTUs less than 3% were classified as rare taxa. Both the rare taxa and the unclassified OTUs were omitted from the plots.

5.2.3 Data analysis

All data were tested with Shapiro-Wilk W for normal distribution prior to analysis. The soil pH, temperature and ecological indices were evaluated statistically by a univariate one-way ANOVA, while the NGS data were analysed as described in 2.12.

5.3 Results

5.3.1 pH changes

The pH values of the control and experimental soils were compared between days 0 (November 2014) and 300 (September 2015) and showed an increase for the piglet treatment on day 14 to reach 7.25 compared to the control (6.69), plant litter (6.75) and piglet + plant litter (6.75) treatments. The piglet treatment recorded a slightly alkaline pH of 7.83 on day 60 while the piglet + plant litter treatment similarly recorded an alkaline value of 7.95 on day 90 in contrast to the control (6.98) and plant litter treatments (6.88). For the piglet and piglet + plant litter treatments, pH decreases resulted between days 120 and 270 to reach values of 6.09 and 5.57, respectively (Figure 5.1). Although temporal changes were recorded, the one-way ANOVA measures for pH values of the control and treatments showed no statistically significant differences ($p = 0.075$) during the study.

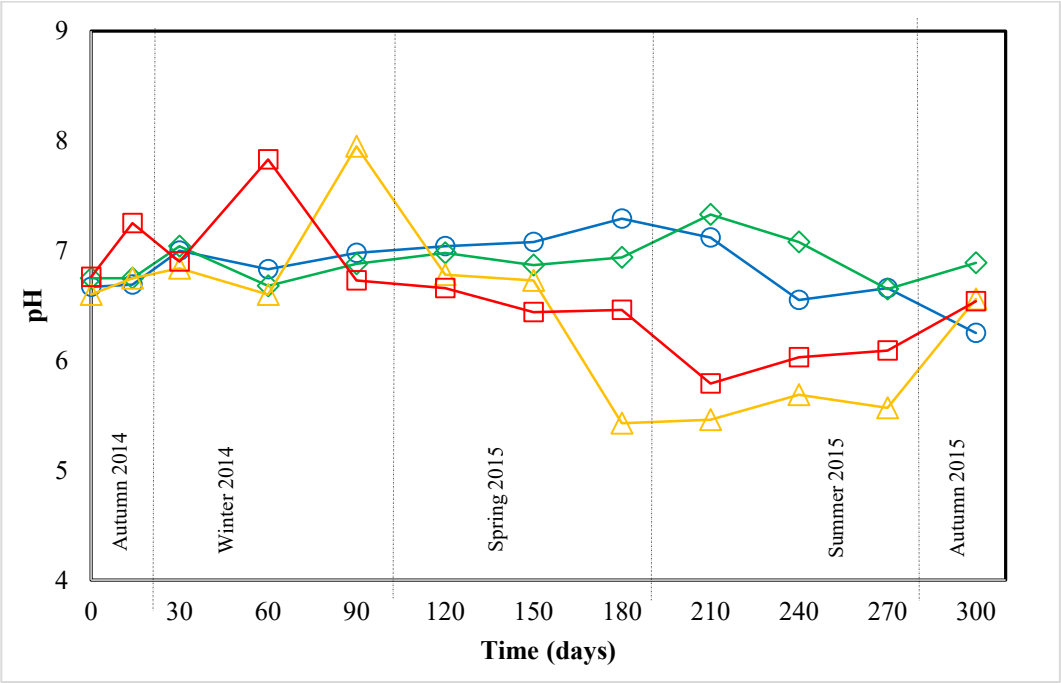


Figure 5.1: Changes in pH values of the control (○), plant litter (◊), piglet (□) and piglet + plant litter (Δ) treatments during 300 days of study.

5.3.2 Temperature

As discussed previously [4.3.2], the ambient, control and treatment soils temperatures were recorded on each sampling day and expressed further as accumulated degree days (ADD) (Table 5.2). Similar profiles were recorded for the control and treatments with temperature increases from day 14 (average ADD 91.5) to day 30 (average ADD 194.1) when the average study soils temperature was 10.7°C. Decreases were then recorded between day 60 (average ADD 310.6) and day 90 (average ADD 382.2) (late autumn to winter) when the average temperature was 5.6°C. A change in season from spring (day 120, average ADD 564.4) to summer (day 240, average ADD 1 929) resulted in an increase in ambient temperature to reach an average of 16.7°C and was followed by falls (day 270, average ADD 2 411.8; day 300, average ADD 2 848.3) to reach an average of 13.6°C (Figure 5.2). The one way-ANOVA showed no statistically significant temporal differences (p = 0.99) between the control and treatments which suggested that any exothermic microbial catabolism temperature increases were negated by the ambient temperature.

Table 5.2: Decomposition temperature timeline as expressed by ADD.

Day	Control	Plant litter	Piglet + Plant litter	Piglet
0	6.3	6.5	6	6.1
14	91.6	92	91	91.2
30	194.1	194.6	193.7	194.1
60	310.7	310.9	310.3	310.4
90	382.6	382.4	381.9	381.9
120	564.8	564.5	564.2	564
150	808.6	807.9	807.6	806.5
180	1 066.6	1 063.2	1 063.8	1 061.6
210	1 466.9	1 462.1	1 463.5	1 460.9
240	1 932.8	1 927.8	1 929	1 926.4
270	2 415.4	2 410.6	2 411.9	2 409.1
300	2 852.4	2 847.2	2 848.3	2 845.3

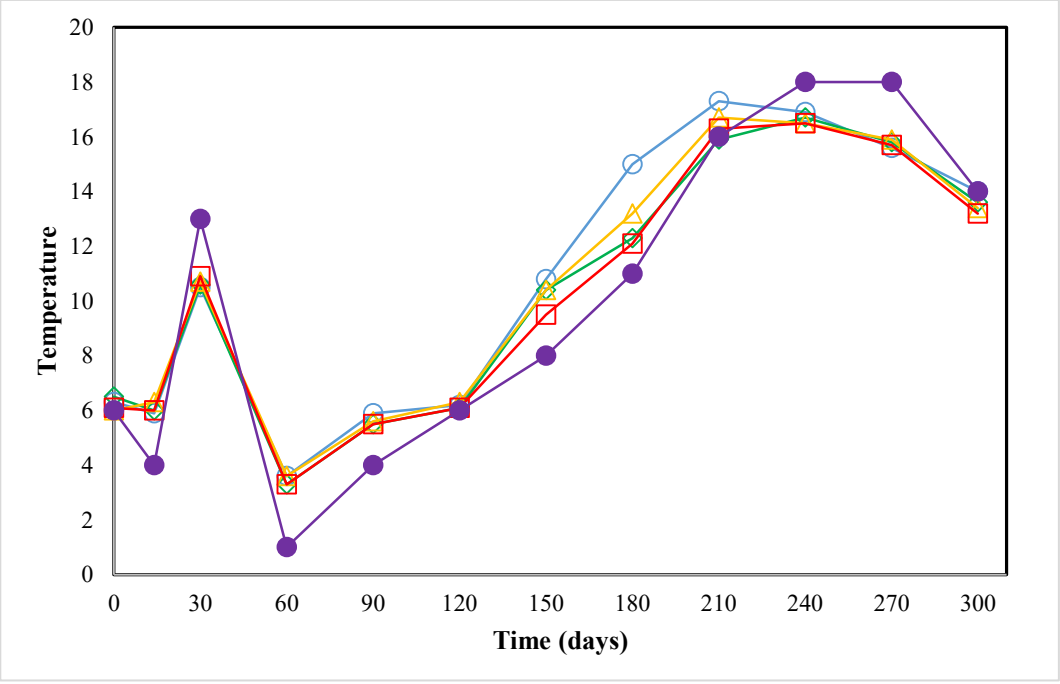


Figure 5.2: Changes in soil temperature (°C) of the control (○), plant litter (◊), piglet (□), piglet + plant litter (△) treatments and ambient temperature (●) during 300 days of study.

5.3.3 Soil ecological analysis

Shifts in soil biodiversity of both 16S bacterial and 18S fungal rRNA gene profiles were analysed by the richness and Shannon-Wiener and Simpson indices [2.11].

Richness

For the 16S rRNA gene richness profiles, similar trends were recorded for the control and experimental treatments between days 0 and 90 (Figure 5.3a). Specifically, periods of increasing richness between days 0 and 30, to values of 27 (control), 28 (plant litter), 25 (plant litter + piglet) and 29 (piglet) were recorded, while those for days 90 to 120 reached 18 (control), 23 (plant litter) and 29 (piglet). These were separated by a phase of decreased richness between days 30 and 90 to reach 16 (control), 15 (plant litter), 16 (piglet + plant litter) and 17 (piglet). Decreases in richness were recorded for the treatments from day 120 to day 150 to reach 15 (plant litter), 14 (piglet + plant litter) and 11 (piglet), which contrasted the control where an increase to 27 between days 120 and 150 was observed. Richness increases were then recorded from day 180 to day 240 when values of 26 (plant litter) and 29 (piglet) were observed in contrast with the control which reached 15 on day 210. Overall, the one way-ANOVA for the 16S bacterial richness changes showed no statistically significant temporal differences ($p = 0.52$) between the control and treatments.

The 18S rRNA gene richness profiles also revealed differences between the control and treatments with contrasting counts first recorded on day 14 with decreases in the control (16) and piglet (11) treatments contrasting an increase to 19 for the plant litter treatment. Increases in richness then resulted from day 30 to day 120 to reach 25 (control) and 22 (plant litter). The piglet treatment decreased in richness from day 180 to day 300 to 15, which contrasted an increase from day 270 to day 300 to 32 for the plant litter treatment (Figure 5.3b). Despite the observed changes, the one way-ANOVA revealed no statistically significant differences ($p = 0.10$) between the control and treatments.

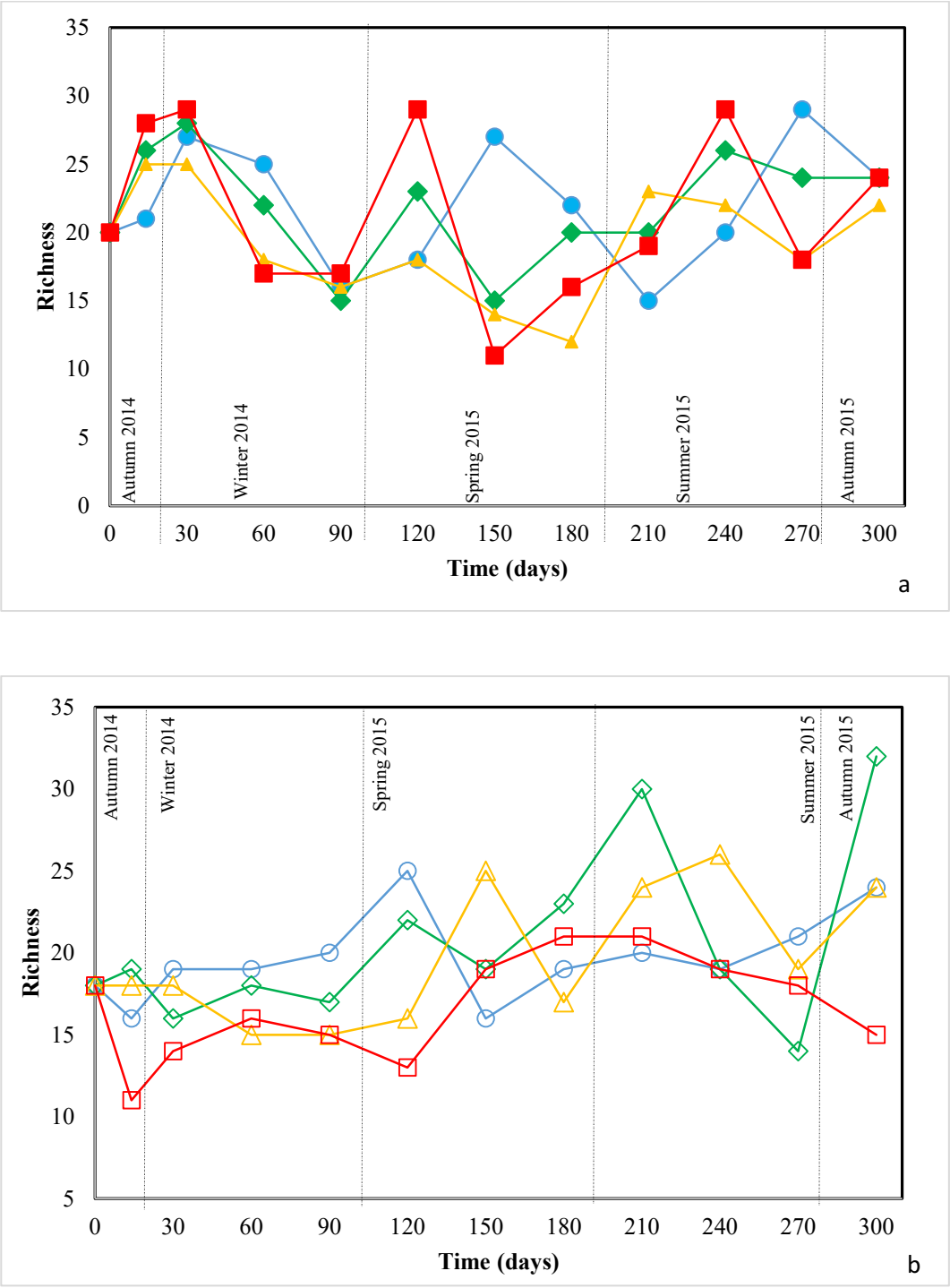


Figure 5.3: 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) richness for control (○), plant litter (◊), piglet (□) and piglet + plant litter (△) treatments during 300 days of study.

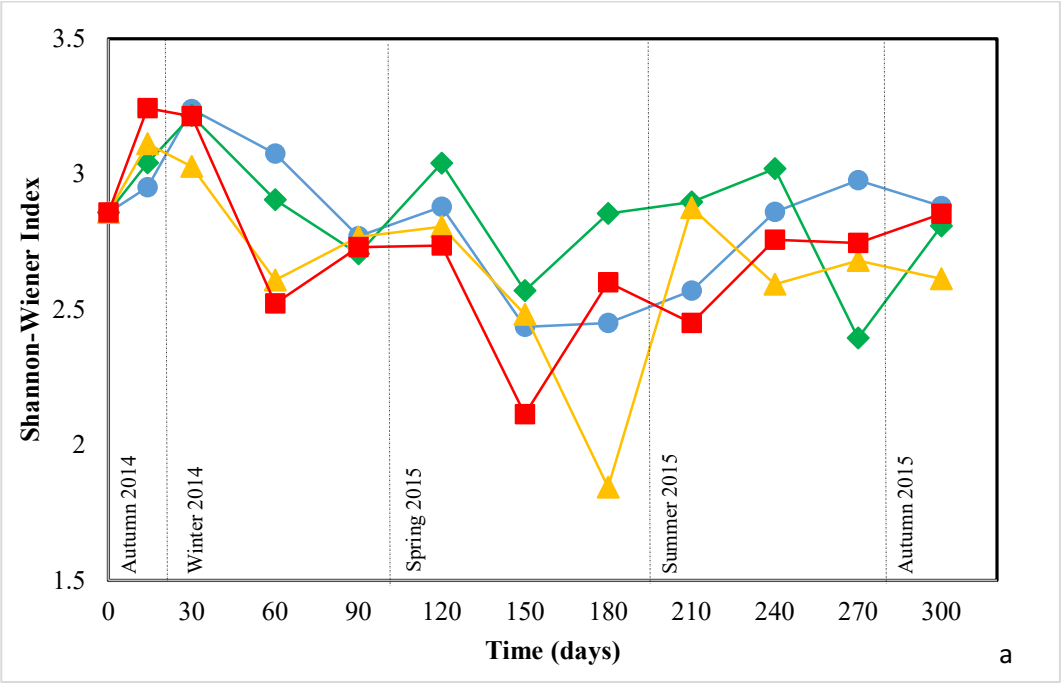
Shannon-Wiener diversity

The bacterial 16S rRNA gene Shannon-Wiener index profiles showed differences in diversity between the control and treatments (Figure 5.4a). Initially, increases were apparent with values of 2.95 (control), 3.04 (plant litter), 3.11 (piglet + plant litter) and 3.24 (piglet)

recorded on day 14. Between days 30 and 150, decreases in the Shannon-Wiener indices were observed with values of 2.44 (control), 2.48 (piglet + plant litter) and 2.11 (piglet) reached. The one exception was the plant litter treatment where an increase to 3.04 resulted between days 90 and 120. For days 150 to 240, the Shannon-Wiener indices of the control and piglet treatment increased to 2.86 and 2.76, respectively while an increase to 3.02 in the same period was followed by a decrease to 2.39 on day 270 in the plant litter treatment.

For the piglet + plant litter treatment, a decrease to 1.84 between days 150 and 180 was followed by an increase to 2.88 on day 210 after which a decrease was again apparent to 2.59 on day 240 (Figure 5.4a). In general, and despite temporal fluctuations, the piglet treatment recorded similar bacterial Shannon-Wiener indices at the beginning (day 0, 2.86) and end (day 300, 2.85) of the study. Also, there were no statistically significant differences ($p = 0.41$) between the control and treatments despite temporal changes.

In contrast to the 16S bacterial dynamics, the 18S fungal Shannon-Wiener indices profiles revealed unique trends and showed statistically significant temporal differences ($p = 0.038$) between the control and treatments (Figure 5.4b). Specifically, there was a decrease in the fungal Shannon-Wiener index for the piglet treatment from day 0 (2.86) to day 60 (2.08), after which a progressive increase was recorded until day 210 (2.62). For the plant litter treatment, a Shannon-Wiener index increase resulted between days 0 and 14 (2.93) and was followed by subsequent decreases until day 90 (2.47). Increases were then recorded between days 150 and 210 to reach 3.14, before falling to 2.28 on day 270. A plateau at approximately $H' = 2.88$ was recorded for the piglet + plant litter treatment during the first 30 days of incubation.



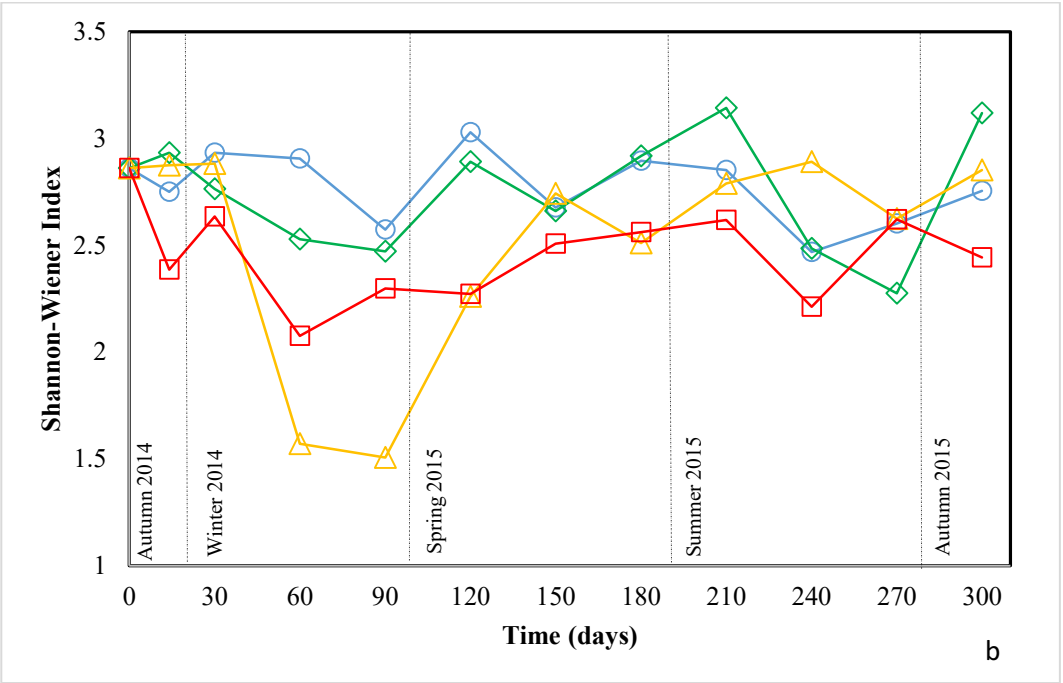


Figure 5.4: 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) Shannon-Wiener indices of the control (○), plant litter (◊), piglet (□) and piglet + plant litter (△) treatments during 300 days of study.

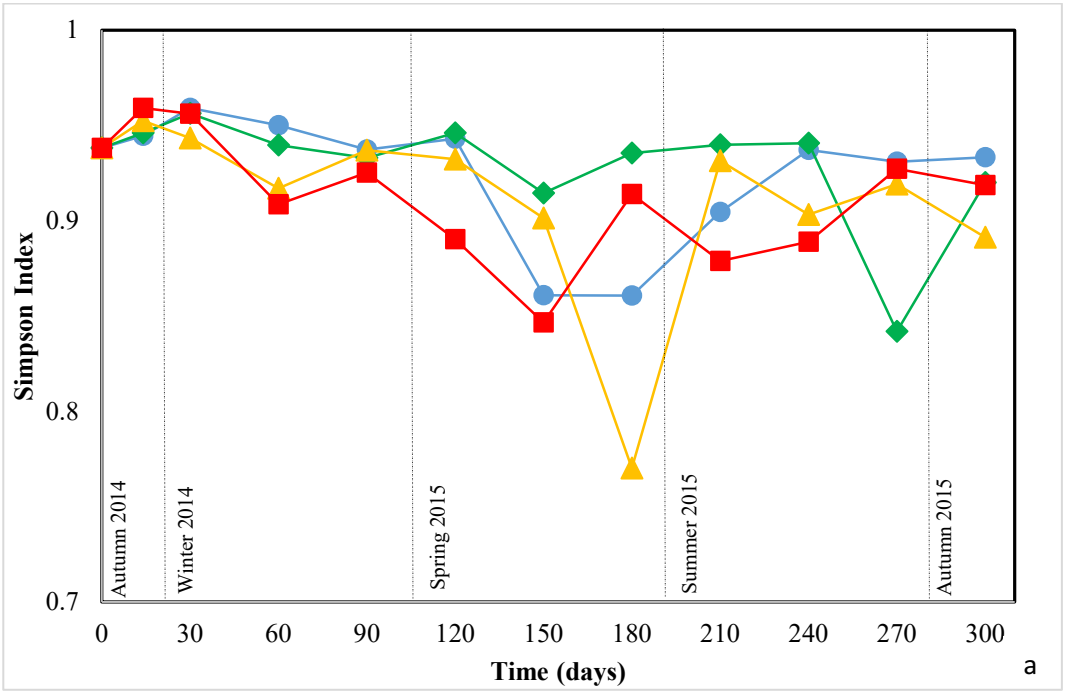
This was followed by a marked decrease in the Shannon-Wiener index between days 30 and 90 (1.51) and increases between days 90 and 150 (2.74) and days 180 and 240 (2.89). For the control a different trend was recorded with decreases from day 30 to day 90 (2.57) and

180 to 240 (2.47) while increases resulted between days 90 and 120 (3.03) and 240 and 300 (2.76).

Simpson diversity

As recorded for the Shannon-Wiener indices, the 16S bacterial Simpson indices also revealed diversity trend differences between the control and treatments (Figure 5.5a). Increases were recorded for all four during the first 14 days with the control and treatments then showing different decreasing rates for the Simpson index between days 30 and 60 (piglet + plant litter, 0.92; piglet, 0.91) and day 90 to day 150 (control, 0.86; piglet, 0.85). A further marked decrease resulted for the piglet + plant litter treatment from day 120 to day 180 to reach 0.77, which was followed by a steep increase to 0.93 on day 210. An increase in the Simpson index was recorded for the control from day 180 to day 240 to reach 0.94 while a decrease to 0.89 resulted between days 210 and 300 for the piglet + plant litter treatment. Overall, the Simpson index did not identify statistically significant differences (p = 0.55) between the control and treatments.

The 18S Simpson indices showed similar trends for fungal community diversity (Figure 5.5b) as the Shannon-Wiener indices (Figure 5.4b). For example, the piglet + plant litter treatment recorded a plateau during the first 30 days of the study and was followed by marked decreases to day 90 (0.62) and subsequent increases by day 150 (0.92) (Figure 5.5b). Also, the control and plant litter treatments recorded similar temporal trends with indices higher than the piglet treatment. Both the control and plant litter treatment indices fell between days 210 and 270 to reach 0.90 and 0.88, respectively in contrast with the piglet treatment which rose between days 240 and 270 to 0.92. Similar to the 16S Simpson index, a one-way ANOVA for the 18S index revealed no statistically significant temporal differences (p = 0.10) between the control and treatments although unique trends were recorded.



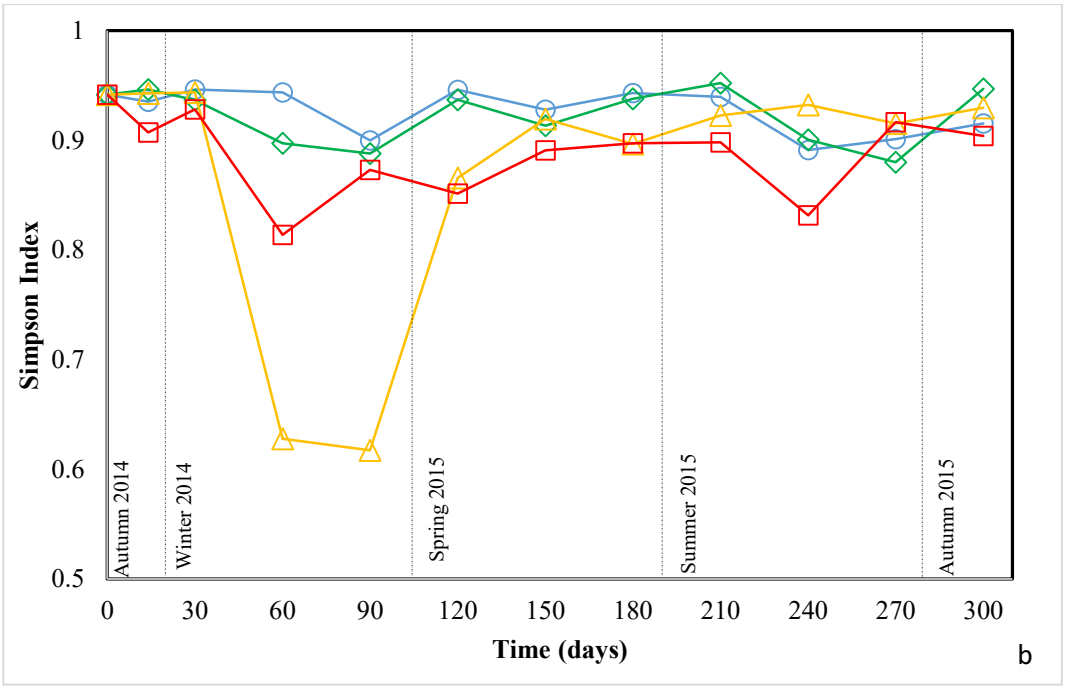


Figure 5.5: 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) Simpson indices of the control (○), plant litter (◊), piglet (□) and piglet + plant litter (Δ) treatments during 300 days of study.

Principal component analysis for ecological measures

The 16S rRNA gene PCA biplot of the Shannon-Wiener (*H'*), Simpson (*D*) and Richness (*S*) ecological measures (Figure 5.6a) showed that PC1 accounted for 53.59% of the variations in the bacterial community dynamics while PC2 accounted for 17.33%. The location of ecological measures along the right axis showed high measures of diversity at the sampling times with no distinct divergences for the control and treatments. For the 18S fungal ecological measures, PC1 accounted for 38.44% of the variations, while PC2 accounted for 24.13% (Figure 5.6b). Also, in contrast to the 16S bacterial ecological measures, the 18S fungal community dynamics clusters for the control and treatments correlated more strongly with each treatment type and corresponding ecological measures. This is highlighted by the delineations for the control, plant litter and pig + plant litter treatments. Overall, the ecological measures of richness, Shannon-Wiener index and Simpson index were highly correlated throughout the study hence evaluation of the 16S bacterial and 18S fungal PC1 and PC2 by ANOVA showed no significant differences ($p < 0.05$).

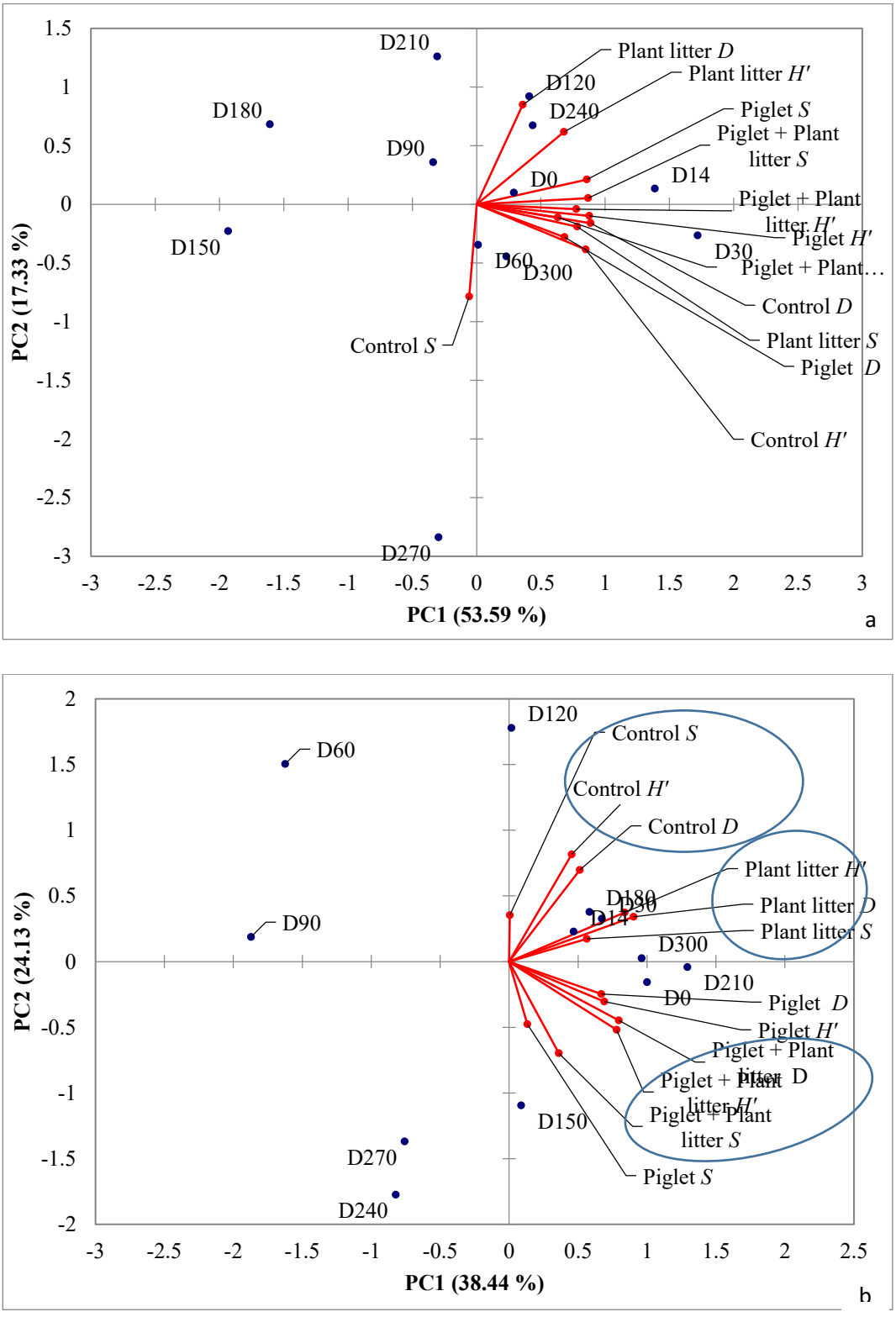


Figure 5.6: 16S bacterial (a) and 18S fungal (b) PCA biplots for ecological measures (Shannon-Wiener, *H′*; Simpson, *D*; Richness, *S*) at specific sampling times of the control, plant litter, piglet + plant litter and piglet treatments.

5.3.4 NGS taxonomic resolution

Phylum-level resolution

The taxonomic resolution at phylum level revealed general dominances in decreasing order of Proteobacteria (18.27 – 56.29%), Acidobacteria (6.07 – 41.60%), Bacteroidetes (3.39 – 28.17%), Verrucomicrobia (3.83 – 18.19%), Actinobacteria (5.26 – 12.92%), Planctomycetes (2.56 – 11.87%) and Firmicutes (1.94 – 10.14%) in the control and experimental treatments (Figure 5.7). Acidobacteria, Planctomycetes, Firmicutes and Verrucomicrobia were found to correlate negatively with temperature while Gemmatimonadetes and Proteobacteria correlated positively. Taxa such as Planctomycetes and Firmicutes correlated positively with pH, while Protebacteria correlated negatively (Table 5.3).

PERMANOVA analysis showed significant differences (p = 0.0001) between the control and treatment soils while the alpha diversity measures, as expressed by Shannon-Wiener index box plot, showed no statistically significant differences (p = 0.06) (Figure 5.8). The spatial distributions, as analysed with NMDS (Figure 5.9), showed taxa similarities with the clustering of the control and treatments from day 0 to day 180 although taxa differences were more pronounced for the piglet treatment from day 150 to day 300. Likewise, the pig + plant litter showed some taxa differences between day 240s and 270 while taxa spatial differences were observed for the plant litter between days 180 and 240. Most of the differences in spatial distribution were observed between the spring and summer periods indicative of season-related changes. Overall, phylum-level dominances did not, generally, provide sufficient differentiation between the absence and presence of the decomposing plant litter, piglet and plant litter + piglet.

Table 5.3: OTUs recording statistically significant correlations ($p < 0.05$) with soil temperature and pH.

Temperature					
Positive			**Negative**		
OTUs (phylum)	**R**	***p***	**OTUs (phylum)**	**R**	***p***
Bacteroidetes	0.342	0.029	Acidobacteria	-0.386	0.013
Gemmatimonadetes	0.518	0.001	Planctomycetes	-0.678	<0.0001
Proteobacteria	0.449	0.004	Verrucomicrobia	-0.498	0.001
			Firmicutes	-0.339	0.031
pH					
Chloroflexi (rare taxon)	0.460	0.003			

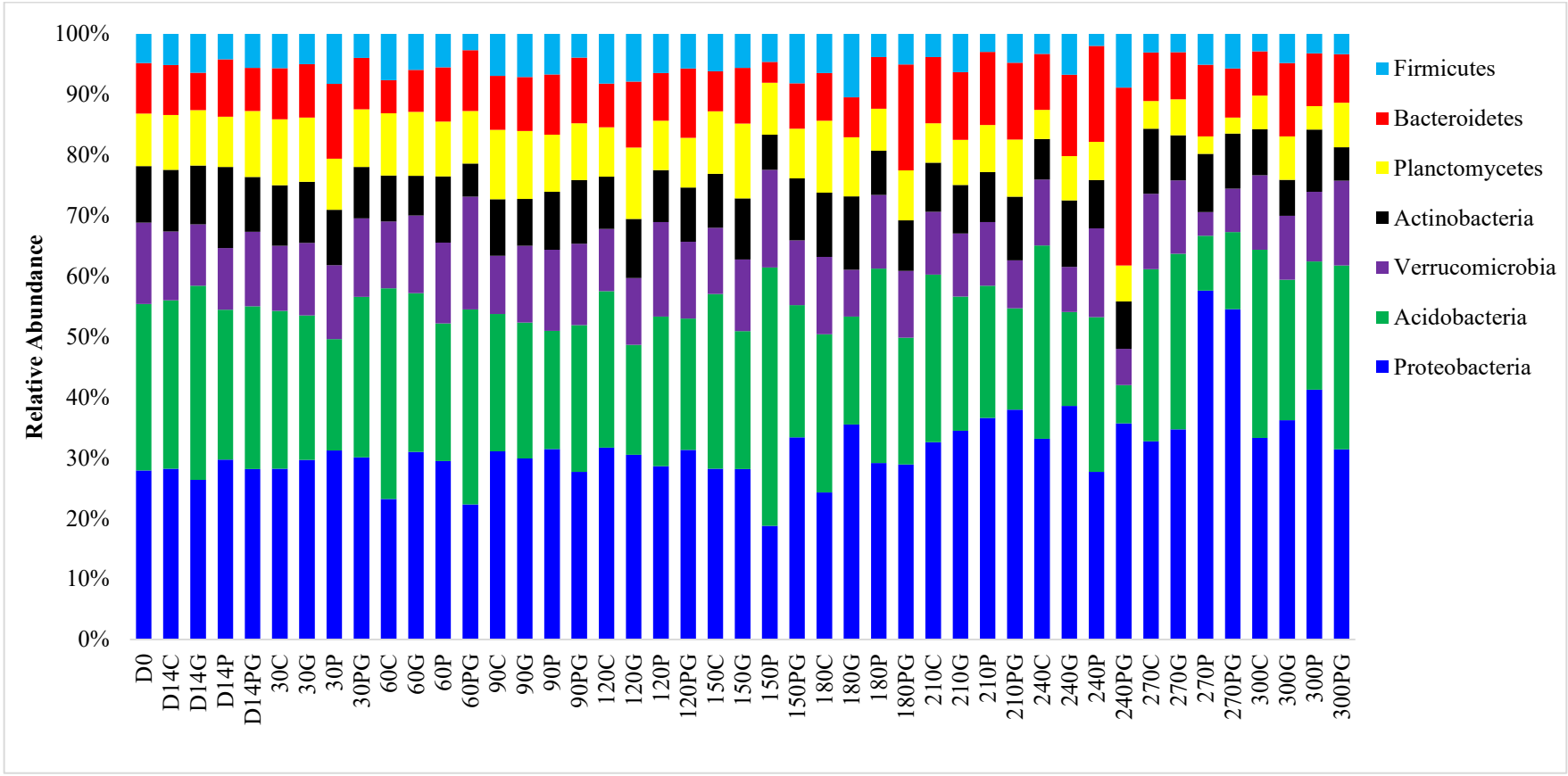


Figure 5.7: Phylum-level bacterial resolution of extracted DNA samples of the control (C), plant litter (G), piglet (P) and piglet + plant litter (PG) treatments.

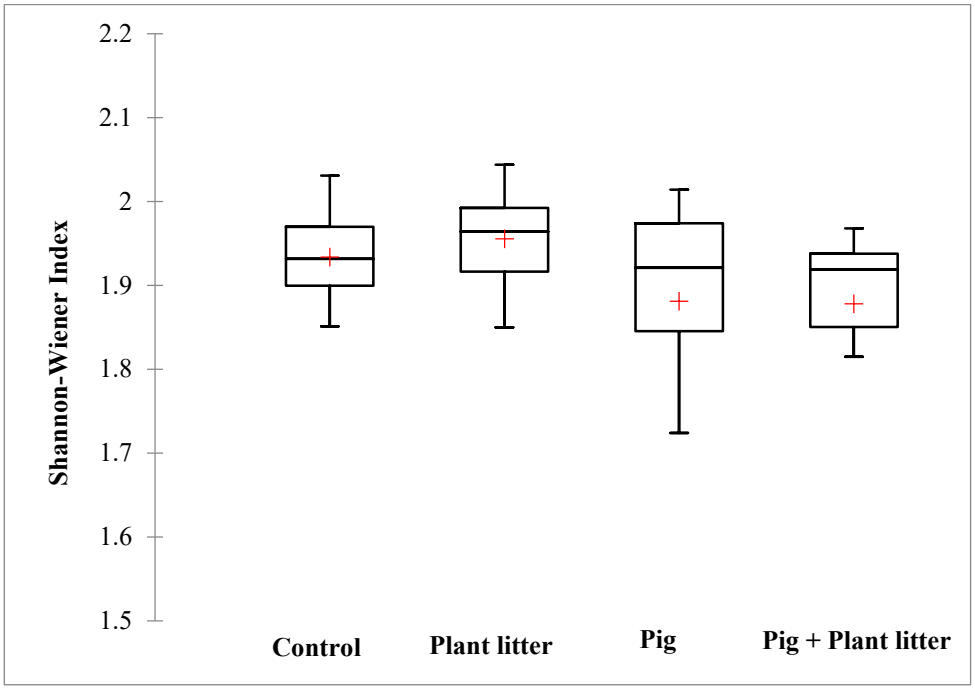


Figure 5.8: 16S bacterial taxa Shannon–Wiener diversity box plot of the control, plant litter, piglet and piglet + plant litter treatments.

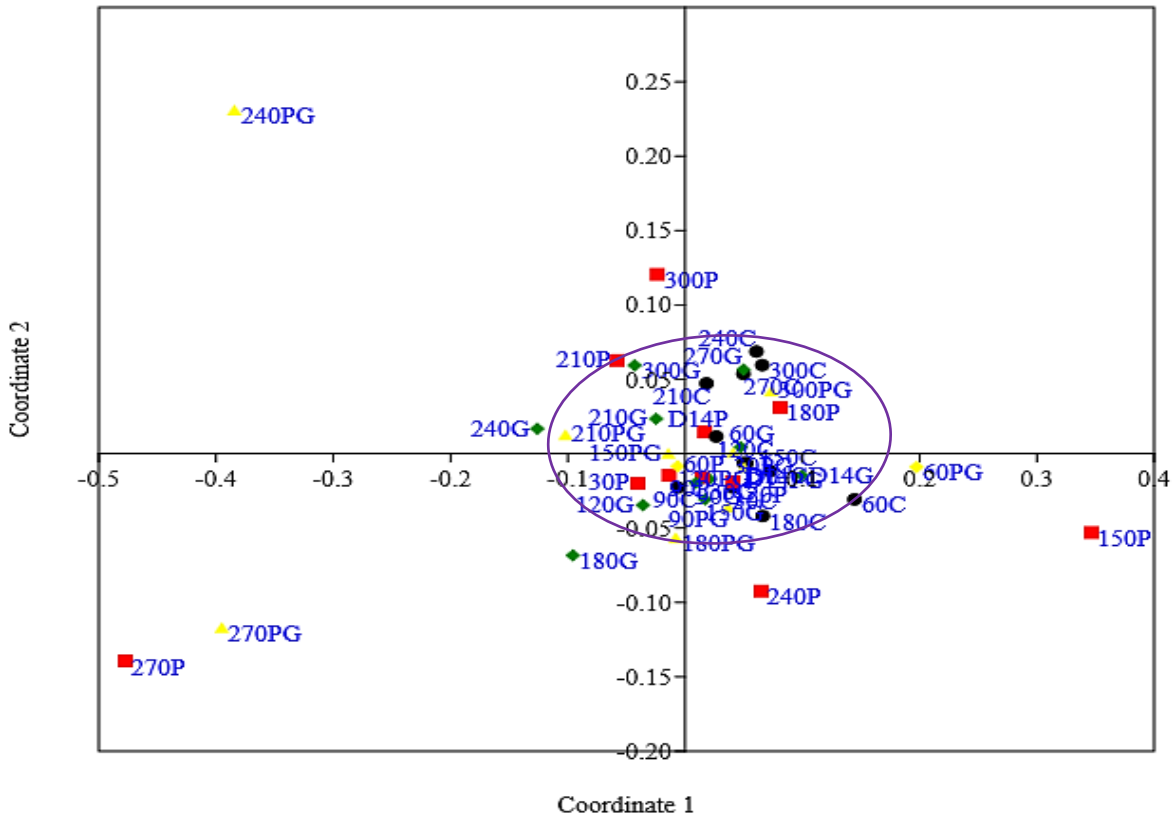


Figure 5.9: NMDS plot (stress = 0.14) of extracted DNA samples of the control (C, ●), plant litter (G, ♦), piglet (P, ■) and piglet + plant litter (PG, ▲) treatments.

Order-level resolution

16S bacterial community resolution at the order level (Figure 5.10) revealed temporal changes between the control and treatments. Between days 0 and 60, the dominant orders for all treatments were Acidobacteria_Gp6_order, Spartobacteria_order and Planctomycetales. Some taxa differences were, however, recorded such as the relative abundance increase of Actinomycetales to 8.58% by day 14 for the piglet treatment and increases of Spartobacteria_order (16.36%) and Flavobacteriales (4.36%), accompanied by a decrease of Rhizobiales (5.42%)for the piglet + plant litter treatment by day 60.

For the control (C), an increase in the relative abundance of Xanthomonadales to 6.55% was apparent on day 120. From day 150 (April 2015), a seasonal change to spring effected a shift in the community taxa composition with increases in the relative abundances of Xanthomonadales (8.74%) and Bacillales (8.01%) for the piglet + plant litter (PG) treatment compared to the other treatments, which for the piglet (P) treatment was characterised by increases in Acidobacteria_Gp6_order (30.09%), Acidobacteria_Gp4_order (7.48%) and Spartobacteria _order (14.94%). On day 180, taxa differences were again recorded with predominances of Xanthomonadales (13.74%) (G), Acidobacteria_Gp4_order (8.47%) and Acidobacteria_Gp3_order (2.87%) (P), and increases in the relative abundances of Flavobacteriales (6.48%) and Bacteriodales (3.93%) (PG). Further changes were observed on day 210 with decreases of Xanthomonadales (8.58%) and Acidobacteria_Gp6 (6.72%) in the plant litter and piglet + plant litter treatments, respectively. In contrast, an increase of Sphingobacteriales was recorded for all treatments (6.84%, C; 10.21%, G; 6.60%, P; 8.77%, PG) with an additional increase of Myxococcales

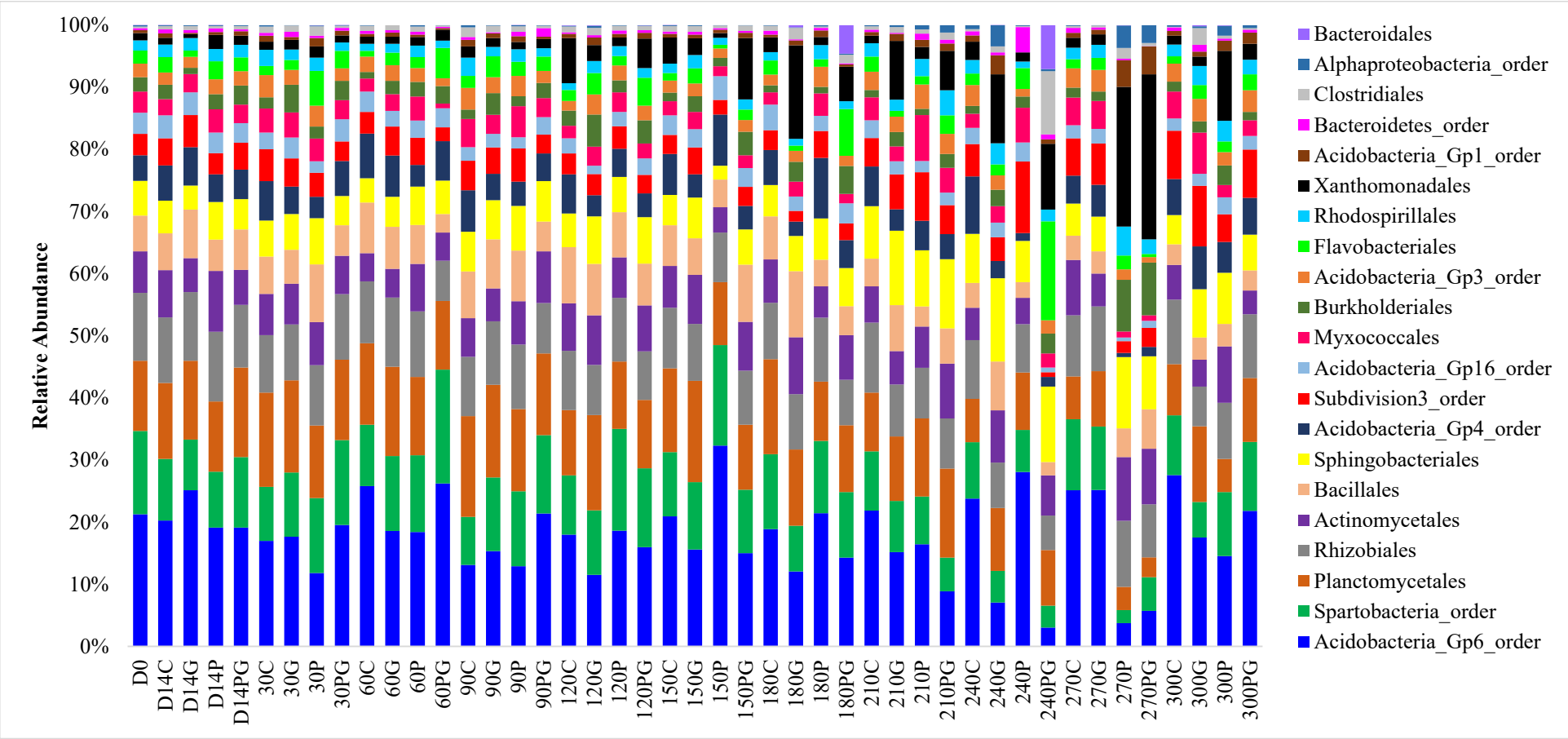


Figure 5.10: Order-level bacterial resolution of extracted DNA samples of the control (C), plant litter (G), piglet (P) and piglet + plant litter (PG) treatments.

(5.83%) in the presence of the piglet while Planctomycetales was the dorminant order of the piglet + plant litter treatment.

Changes in the epinecrotic microbial communities were seen on day 240 with increases in taxa abundances of Flavobacteriales (11.07%), Sphingobacteriales (10.32%), Clostridiales (8.03%), Bacteroidales (5.55%) and Xanthomonadales (7.92%) in the piglet + plant litter treatment, where they became the dominant orders. While the piglet treatment recorded increases in Acidobacteria_Gp6_order (24.56%), Subdivision3_order (9.88%), Myxococcales (4.72%) and Bacteriodetes_order (3.20%), the plant litter treatment recorded a decrease in Acidobacteria_Gp6_order (6.50%) and increases in Acidobacteria_Gp1_order (2.79%) and Alphaproteobacteria_order (2.77%). Both the piglet and piglet + plant litter treatments recorded similar taxa shifts with increases in the relative abundances of Xanthomonadales (P, 20.44%; PG, 24.75%), Burkholderiales (P, 7.59%; PG, 7.45%) and Acidobacteria_Gp1_order (P, 3.50%; PG, 4.09%) and decreases in Planctomycetales (P, 3.24%; PG, 2.89%). In contrast, Acidobacteria_Gp6 was predominant for the control and plant litter treatments. Finally, Xanthomonadales decreases (P, 9.99%; PG, 2.25%) and increases of Acidobacteria_Gp6_order (P, 12.71%; PG, 19.01%), Planctomycetales (P, 4.58%; PG, 9.01%) and Spartobacteria_order (P, 8.73%; PG, 9.11%) were recorded on day 300 whereas increased relative abundances of Subdivision3_order (C, 6.79%; G, 7.57%) were recorded.

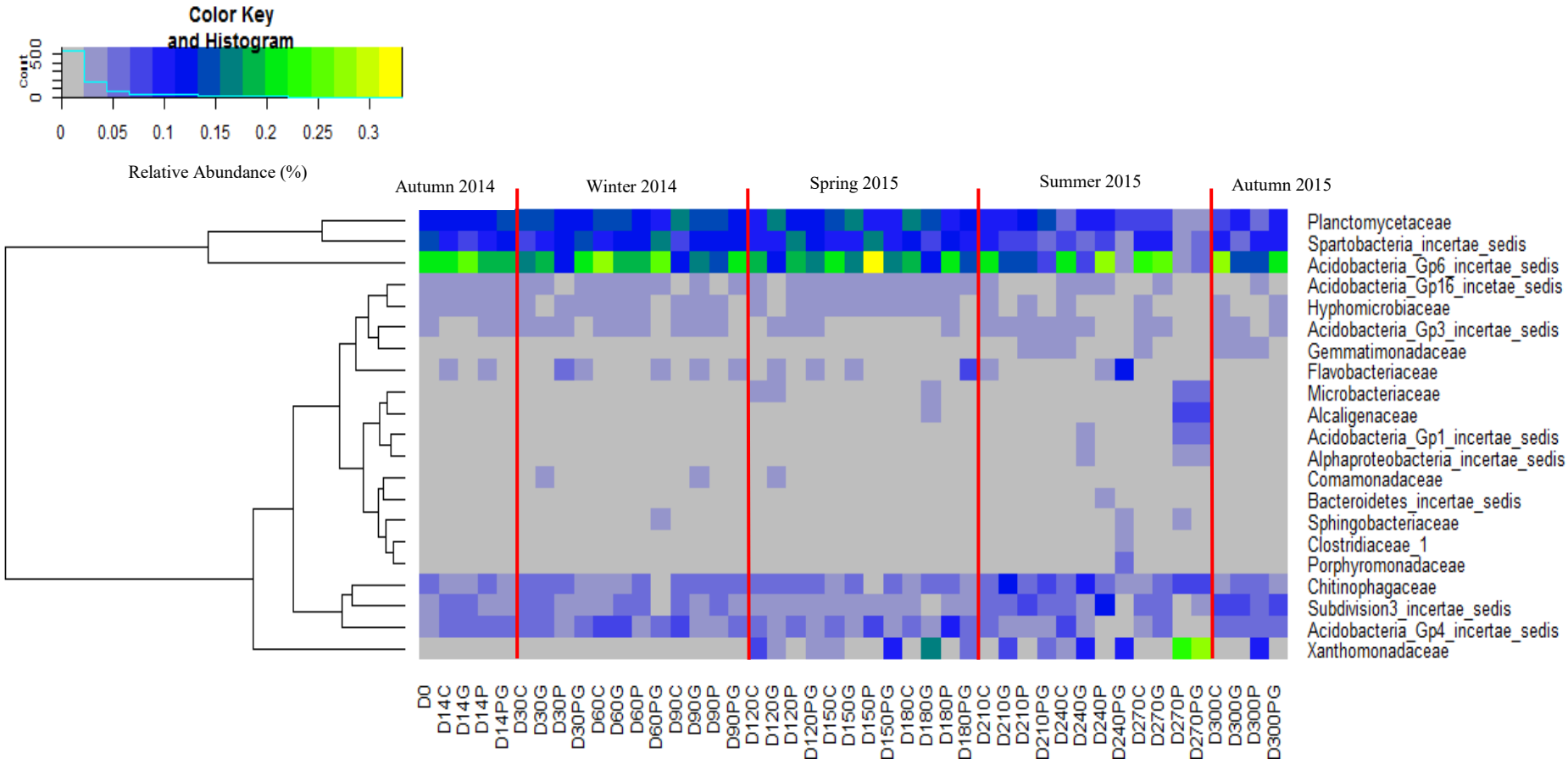


Figure 5.11: Heatmap to visualise the relative abundances (%) of the most predominant bacterial families (>0.3%) for the control (C), plant litter (G), plant litter + piglet (PG) and piglet (P) soils.

Family-level resolution

A heatmap (Figure 5.11) further visualised and identified key microbial taxa and showed distinct changes. Comamonadaceae, aerobic heterotropic bacteria that are mostly free-living saprophytes (Willems 2014) dominated the plant litter treatment on days 30, 90 and 120 while Sphingobacteriaceae, Gram-negative spore-forming bacilli mostly found in soil (Lambiase 2014), were observed for the pig + plant litter treatment on days 60 and 240 and piglet treatment on day 270.

Seasonal changes resulted in dominance of taxa such as Microbacteriaceae, Alcaligenaceae, Acidobacteria_Gp1, and Alphaproteobacteria_incertae for both piglet and piglet + plant litter treatments on day 270. In constrast, Acidobacteria_Gp1 and Alphaproteobacteria_incertae were observed for the plant litter on day 240 whilePorphyromonadaceae and Clostridiaceae dominated the piglet + plant litter treatment on the same day. Increase in the relative abundance of Flavobacteriaceae was recorded for the piglet + plant litter treatment between days 180 and 240. Gemmatimonadaceae, Gram-negative aerobes (Hanada and Sekiguchi, 2014), were observed from day 210 for the control and treatments. The dominance of Xanthomonadaceae, Gram-negative denitrifying bacteria (Green *et al*., 2012), was observed for the piglet and piglet + plant litter treatments on day 270. For most of the families, the changes were more pronounced during the spring and summer periods.

Pairwise comparison with Turkey *post hoc* identified OTUs with statistically significant differences at family-level resolution between the control and treatment soils (Table 5.4) and relative to season (Table 5.5). For example, Comamonadaceae and Planctomycetaceae recorded significant differences ($p < 0.05$) between the pig and plant litter treatments whereas Hyphomicrobiaceae was observed to be significantly different ($p < 0.0001$) between the control and piglet + plant litter treatment. Likewise, significant seasonal differences ($p < 0.05$) were observed with OTUs such as Chitinophagaceae and

Alphaproteobacteria_incertae observed for the 2015 summer. Furthermore, both Gemmatimonadaceae and Planctomycetaceae were observed to be significantly different (p <0.05) in summer 2015 and autumn 2015 compared to autumn 2014, winter 2014 and spring 2015 while The family Comamonadaceae was significantly different ($p < 0.006$) between winter 2014 and summer 2015.

Table 5.4: Family-level OTUs that are statistically significantly different between the control and treatments soils according to the least squares means (LS-means). Combinations sharing the same letter (a, b) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTU family	Control	Plant litter	Pig	Pig + Plant litter	*p*
Acidobacteria_Gp4	0.062 a	0.045 ab	0.045 ab	0.042 b	0.011
Comamonadaceae	0.004 b	0.012 a	0.003 b	0.009 ab	0.006
Hyphomicrobiaceae	0.028 a	0.024 ab	0.023 ab	0.021 b	< 0.0001
Planctomycetaceae	0.116 ab	0.130 a	0.103 b	0.109 ab	< 0.0001

Table 5.5: Family-level OTUs that are statistically significantly different between seasons according to LS-means. Combinations sharing the same letter (a, b, c) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTU family	Autumn2014	Winter2014	Spring2015	Summer2015	Autumn2015	*p*
Acidobacteria_Gp16	0.035 a	0.027 b	0.029 ab	0.019 c	0.021 bc	0.000
Alphaproteobacteria	0.002 b	0.001 b	0.001 b	0.011 a	0.006 ab	0.029
Chitinophagaceae	0.045 b	0.044 b	0.046 b	0.065 a	0.047 ab	0.006
Comamonadaceae	0.006 ab	0.011 a	0.008 ab	0.003 b	0.004 ab	0.006
Gemmatimonadaceae	0.010 b	0.012 b	0.011 b	0.019 a	0.025 a	< 0.0001
Hyphomicrobiaceae	0.031 a	0.026 ab	0.026 ab	0.017 c	0.018 bc	< 0.0001
Planctomycetaceae	0.123 a	0.138 a	0.125 a	0.085 b	0.085 b	< 0.0001
Spartobacteria	0.118 a	0.119 a	0.118 a	0.068 b	0.088 ab	0.000
Subdivision3	0.040 b	0.041 b	0.032 b	0.050 ab	0.070 a	0.003

5.4 Discussion

pH and temperature

An increase in piglet gravesoil pH between days 14 and 60 was in agreement with earlier reports by Haslam and Tibbett (2009) and Meyer *et al*. (2013) where the initial increase was attributed to mineralisation of base-forming cations (Ca^{2+}, K^{+} and Mg^{2+}) and ammonification of organic nitrogen (proteins and peptides). The subsequent decrease was possibly due to nitrate accumulation (Meyer *et al*., 2013). The differences in pH recorded for the piglet + plant litter treatment, when compared to both the piglet and plant litter only treatments, can be attributed to changes in soil microbial metabolic and activity rates in relation to catabolic products where the *S. scrofa domesticus* contributed proteins, lipids and carbohydrates (Janaway *et al*., 2009) while plant litter largely contributed cellulose and lignin (Tagliavini *et al*., 2007).

Differences in ambient and treatment temperature can be attributed directly to seasonal weather changes and exothermic microbial catabolism. For this study, the seasonal shifts occurred for autumn 2014 (days 0 to 30), winter (days 30 to 120), spring (days 120 to 210), summer (days 210 to 270) and autumn 2015 (days 270 to 300). As observed in this study, seasonal temperature shifts resulted in microbial community structure changes as expressed by ecological indices measurements. As reported by Meyer *et al*. (2013), seasonal differences between winter and summer have considerable impacts on microbial catabolic rates, with reduced and increased rates resulting in winter and summer, respectively. Temperature-mediated microbial activity was discussed by Mann *et al*. (1990) and Zhou and Byard (2011) where an optimum temperature range of 21 to 38°C was reported to enhance microbial activity, while limited activity was recorded at temperatures below 4°C. The highest temperature between the control and the treatments was recorded on day 210/summer 2015 although no significant differences ($p > 0.05$) were recorded along the decomposition timeline.

DGGE-based ecological indices

In general, the ecological measures revealed trends that fluctuated with the decomposition timeline in the control and treatments, which were linked to seasonal temperature shifts. For example, both 16S and 18S ecological trends for the control, plant litter and piglet treatments showed decreased microbial diversity between days 14 and 90 (autumn to mid-winter, 2014) while those of the piglet + plant litter treatment plateaued between days 0 and 30 (autumn 2014). Overall, there was no clear and consistent differentiation between the control and treatments, or between the piglet and plant litter, for the used soil type. The exceptions were for the piglet plus plant litter where both the bacterial 16S and fungal 18S rRNA genes recorded decreased diversities on day 180 (16S) and days 60 and 90 (18S).

Community profiling tools such as DGGE continue to have a role to play in microbial ecology investigations, provided that their caveats are recognized and mitigated for. Also, the relevance of attendant richness, diversity and evenness measurements has been well-established. Therefore, more replicated subsurface studies, with whole cadavers or human surrogates, should be used to investigate the applicability and utility of ecological indices analyses in enhanced PMI and time-since-burial determinations. Whenever possible, these can be complemented with high-throughput analyses.

16S bacterial community taxonomic resolution

Postmortem microbial changes are gaining considerable attention with studies of cadaver decomposition epinecrotic communities (Metcalf *et al*., 2013; Hyde *et al*., 2015; Weiss *et al*., 2016). While characteristically predominant in soils (Roesch *et al*., 2007; Peralta *et al*., 2013; Ligi *et al*., 2014; Arroyo *et al*., 2015), the numerical abundances of Proteobacteria, Acidobacteria and Actinobacteria specifically in decomposition-impacted soils have been reported by various researchers (Carter *et al*., 2015; Damann *et al*., 2015; Finlay *et al*., 2016). Furthermore, the dominance of Verrucomicrobia (Spartobacteria_family and Subdivision3_family) has been reported by Bergmann *et al*. (2011) who also observed

underestimation in soil metagenomics due to primer bias. The dominances of Acidobacteria and Verrucomicrobia observed in this study aligned this investigation to the work of Finley *et al*. (2016) who also reported their numerical abundances in an underground decomposition study when compared to its aboveground equivalent.

Bray-Curtis dissimilarity with nonmetric dimensional scaling (NMDS) for the samples showed that the control soils were similar while differences were observed for the plant litter, piglet and piglet + plant litter treatments. Differences in the treatments were probably due to seasonal temperature changes that were apparent from day 210 to day 270 (summer). Although the phylum-level resolution could, potentially, identify season-based differences for this soil, the dominances recorded did not, generally, provide sufficient composition-based community clustering or differentiation between the mammalian proxy decomposition and the control or plant litter soils.

The numerical dominances on day 270 of Alphaproteobacteria_order (P, 3.30%; PG, 2.65%) and Acidobacteria_Gp1_order (P, 3.93%; PG, 4.18%), above 'background' occurrences at other sampling times, identified them as potential indicators for piglet and piglet + plant litter decomposition with community structure as the target variable. The differential dominance of Xanthamonadales seemed to distinguish the early (days 0 to 90), mid (days 120 to 210), and late (day 270) time-since-burial intervals for the pig + plant litter treatment, in particular. Nevertheless, this order was not necessarily useful for differentiating between: soil containing a decomposing mammalian taphonomic proxy and its control; and piglet and plant litter.

For this current study, it was anticipated that the mixture of piglet and plant litter would effect differences in the epinecrotic communities when compared to the individual components. Days 240 to 270 (summer, July – August 2015) were, however, the only period when considerable differences were recorded with increases of Gram-negative non-motile rod- shaped Porphyromonadaceae (Krieg *et al*., 2010), often described as faecal bacteria

(Newton *et al*., 2013), and obligate anaerobic rod-shaped Gram-positive Clostridiaceae_1 (De-Vos *et al*., 2009) for the piglet + plant litter treatment observed on day 240. Further shifts with the dominance of Gram-positive Microbacteriaceae (Goodfellow *et al*., 2012) and Alcaligenaceae, aerobic Gram-negative rod or coccobacilli chemoorganotrophic bacteria (Whiteson *et al*., 2014), were then recorded for the piglet and piglet + plant litter treatments on day 270. Likewise, Metcalf *et al*. (2013) associated Alcaligenaceae with advanced stages of decomposition.

Some family-level resolutions identified more detailed phylogenetic variations between the treatment soils relative to time. For example, resolution of the order Sphingobacteriales revealed two dominant families, Sphingobacteriaceae and Chitinophagaceae, with the latter consisting of aerobic or facultative anaerobic rod-shaped bacteria (Krieg *et al*., 2010), whose functions include limited fermentative capabilities of carbohydrates and the possession of Menaquinones MK-7. While the dominance of Chitinophagaceae was observed for all of the treatments from day 0 to day 300, Sphingobacteriaceae predominated in the piglet + plant litter treatment on days 60, 120 and 240. This contrasted the piglet treatment, in particular, where increased abundance was only observed on day 270 (summer 2015). Therefore, potentially, the family could be used as a summer microbial clock indicator for pig (human) burials for this soil type. Metcalf *et al*. (2013) and Carter *et al*. (2015) identified Sphingobacteriaceae as a potential microbial indicator while the former researchers aligned this bacterial group with advanced stages of decomposition. Also, the predominance of Chitinophagaceae from day 0 to day 300 (November 2014 – September 2015) contrasted the work of Carter *et al.* (2015) who observed flushes of *Psychrobacter* spp and Chintinophagaceae in winter and summer months, respectively. Therefore, the differences between the findings of this study and those reported in the literature may be, in part, due to the used mixture of piglet + plant litter.

Since soil is heterogeneous, differences in taxa were expected although these were subsequently more pronounced coincident with a seasonal temperature change from spring to summer. For example, both the control and piglet + plant litter treatment recorded an increase in the relative abundance of Xanthomonadaceae (*Rhodanobacter* sp., a Gram-negative denitrifying bacterium; Green *et al*., 2012), on day 120, which might, potentially, be a useful indicator of seasonal change for this soil. Furthermore, Xanthomonadaceae (*Rhodanobacter* sp.) was observed to be the most abundant taxon on day 270 (summer, August 2015) for both the piglet and piglet + plant litter treatment, and so might be used as a time-specific indicator in the postmortem microbial clock.

Both Microbacteriaceae and Alcaligenaceae were recorded for the piglet and piglet + plant litter on day 270 (late summer, August 2015) with Xanthomonadaceae (*Rhodanobacter* sp.) observed to be the most abundant taxon for both the piglet and piglet + plant litter treatments. Therefore, six families (Alcaligenaceae, Chitinophagaceae, Microbacteriaceae, Porphyromonadaceae, Sphingobacteriaceae and, particularly, Xanthomonadaceae (*Rhodanobacter* sp.) could, potentially, be used as seasonal and time-since-burial indicators, *re* microbial community structure and composition, for the human taphonomic proxy, either individually or in combination with plant litter.

Overall analyses of the decomposition timeline identified Comamonadaceae, aerobic Gram-negative bacteria commonly found in soil and water habitats (Willens, 2014), and Planctomycetaceae, aerobic heterotrophic Gram-negative bacteria (Youssef and Elshahed, 2014), as significantly different ($p < 0.05$) between the plant litter and piglet treatments. Also, Hyphomicrobiaceae, aerobic Gram-negative bacteria found in different soil types and water habitats (Oren and Xu, 2014), was significantly different ($p < 0.05$) between the control and pig + plant litter treatment. No taxa were found to be significantly different ($p = 0.05$) between the individual additions and the mixture. For season, differences were recorded mostly during summer 2015 with taxa such as Chintinophagaceae,

Gemmatimonadaceae, Hyphomicrobiaceae and Planctomycetaceae significantly different ($p < 0.05$) when compared to autumn 2014, winter 2014 and spring 2015.

5.5 Conclusions

Abiotic factors such as temperature and pH are key variables that must be used parallel to microbial community profiles to establish and validate a robust postmortem interval estimation tool (Lauber *et al*., 2014; Weiss *et al*., 2016). As observed from the trends in the current study, temperature differences were related directly to seasonal changes in relation to substrate compositions. Results from the study suggested that seasonal temperature changes, which were more pronounced during the summer, led to shifts in microbial community composition as expressed by the ecological indices measurements and next-generation sequencing. For example, 16S bacterial ecological measures revealed distinct changes between spring and summer while 18S fungal ecological measures changes were more pronounced in winter and summer. Therefore, Hypotheses 1 and 2 were accepted relative to specific microbial groups when the ecological indices of richness, Shannon-Wiener and Simpson diversity were used, as also recorded in Chapter 4.

Distinguishing between the soils of the individual treatments and the control based on microbial community profiling is a challenge due to presence of several common dominant taxa such as Proteobacteria, Acidobacteria, Verrucomicrobia, Bacteroidetes, Planctomycetes and Actinobacteria. Of the three programme hypotheses [1.10], the first and third were rejected at phylum level resolution since only the abundance of specific phyla (structure) changed temporally and in response to decomposition.

A Shannon-Wiener diversity box plot revealed no statistically significant differences ($p = 0.06$) between the control and treatments when order-level taxonomic resolution was used. Notwithstanding this, the presence and absence of Bacteroidales, as well as the shifts in the numerical dominances of this and Clostridiales, identified them as possible order-level time-since-burial indicators for the mixed carbon sources. Therefore, Hypotheses 1 – 3, regarding

community composition, structure and unique taxa, respectively, were accepted for this study soil and treatment, at the order level of resolution. Similarly, all three hypotheses were accepted at family-level profiling where some community composition delianation existed between the control and piglet + plant litter treatment due to the occurrence and increased abundance of Porphyromonadaceae. Also, the numerical abundances of several members such as Microbacteriaceae, Flavobacteriaceae, Alcaligenaceae and Xanthomonadaceae identified them as potential community structure-based indicators to differentiate between the control/plant litter and piglet/piglet + plant litter treatments on days 240 and 270 (summer 2015). The use of the pairwise comparison, however, did not identify any significant differences between the individual additions and the mixture. While the piglet + plant litter treatment effected pH change compared to the individual components, only a time-specific analysis on day 240 revealed Porphyromonadaceae and Clostridiaceae_1 as potential temporal taxa indicators to differentiate between the individuals and the combination. Overall seasonal analysis revealed that most of the significant taxa, such as Chintinophagaceae, Gemmatimonadaceae and Hyphomicrobiaceae associated with summer 2015, were aerobic Gram-negative bacteria.

Correlation of ecological measures with next-generation sequencing taxonomic resolution remains a challenge because of the limitations of the DGGE technique [1.9.2] (Green *et al*., 2009; Crippen *et al*., 2015). Notwithstanding this, some temporal differences recorded with the DGGE-dependent ecological measures between the control and treatments were also observed in the NGS-based taxonomic classification during the summer period. Caution was, however, taken not to align these differences directly since the DGGE bands were not excised and subjected to sequencing.

Overall, the presence, absence and relative abundance of taxa, as parameters to identify potential microbial clock and time-since-burial indicators, depended on the level of resolution. Although differences for most taxa within and between treatments seemed to be

less dependent on the presence of any decomposing material or the presence of piglet as the human taphonomic proxy, in particular, some members could be targeted, potentially, relative to seasonal temperature changes especially for mid- to late summer 2015 (days 240 – 270).

Charneca *et al*. (2010) reported a high protein to lipid ratio in newborn piglets, which countered the assumption that the stillborn piglet carcasses used in this study contained relatively high lipid contents. Therefore, future studies should investigate the effects of age and nutrient composition of the animal model on the environmental/soil microbial community dynamics. These comparative studies would not only facilitate experimental database development but also provide information with potential relevance to investigations of suspected clandestine child burials in contrast to those of adults. In summary, additional, multiple and replicated subsurface investigations should resolve further the decomposition-specific taxa occurrences/dominances and changes in microbial community profiles as recorded in the current study and similar future initiatives. Also, these future studies should consider different soil types, plant litter, climatic conditions, geographical regions and effects of freezing conditions of the study animal model in relation to the impacts of its indigenous microbiome and rates of decomposition.

Chapter 6: *In situ* decomposition study[5]

6.1 Introduction

As discussed in Chapter 1, microorganisms play crucial roles in cadaver decomposition (Metcalf *et al*., 2016; Weiss *et al*., 2016) so there is intense interest in elucidating the relationships between cadaver microbiota and soil microbial fauna as potential indicators in forensic applications. For example, the possible use of the epinecrotic community as a "postmortem microbial clock" was proposed by Metcalf *et al*. (2013) and Pechal *et al*. (2014). In a recent study, Metcalf *et al*. (2016) stated that approximately 40% of microbial decomposer communities were found at very low abundances in soils at the start of their experiments and recorded significant changes in microbial communities relative to seasonal shifts (spring and winter). Notwithstanding this, comparisons between soil microbial communities associated with subsurface cadaver and leaf decomposition processes remain unexplored [Table 1.1].

To address this knowledge gap, a study was made with stillborn piglets and deciduous leaf litter (*Quercus robur*) to address the the three research programme hypotheseses *in situ* as follows:

1. *In situ* subsurface decomposition of whole *Sus scrofa domesticus* as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.
2. *In situ* subsurface decomposition of whole *S. scrofa domesticus* will effect different shifts in the surrounding soil microbiome structure and composition when compared to leaf (*Quercus robur*) litter.

[5] A substantive proportion of this work was published as follows:

Olakanye A.O., Nelson A. and Ralebitso-Senior T.K. 2017. A comparative *in situ* decomposition study using still born piglets and leaf litter from a deciduous forest. *Forensic Science International* **276**: 85–92.

3. Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and leaf litter (*Quercus robur*) decompositions.

6.2 Experimental design

6.2.1 The study

Prior to the study, the well-secured site, located at Monk Fryston, North Yorkshire, U.K. (Lat. 53.76°N, Long. 1.23°W), was cleared of vegetation and mapped with a Leica GS15 global navigation satellite system (GNSS; Heerbrugg, Switzerland) with real-time kinematic (RTK) corrections providing, typically, 10 mm accuracy. The soil was characterised as a loam constituted by (w/w) 22% clay, 32% silt and 46% sand and physicochemical characteristics of nitrate aqueous extract as NO_3^- (3.5 mg l^{-1}), total organic carbon (3.0%), total S (0.03%), pH (7.9) and P (<0.10 mg kg^{-1}).

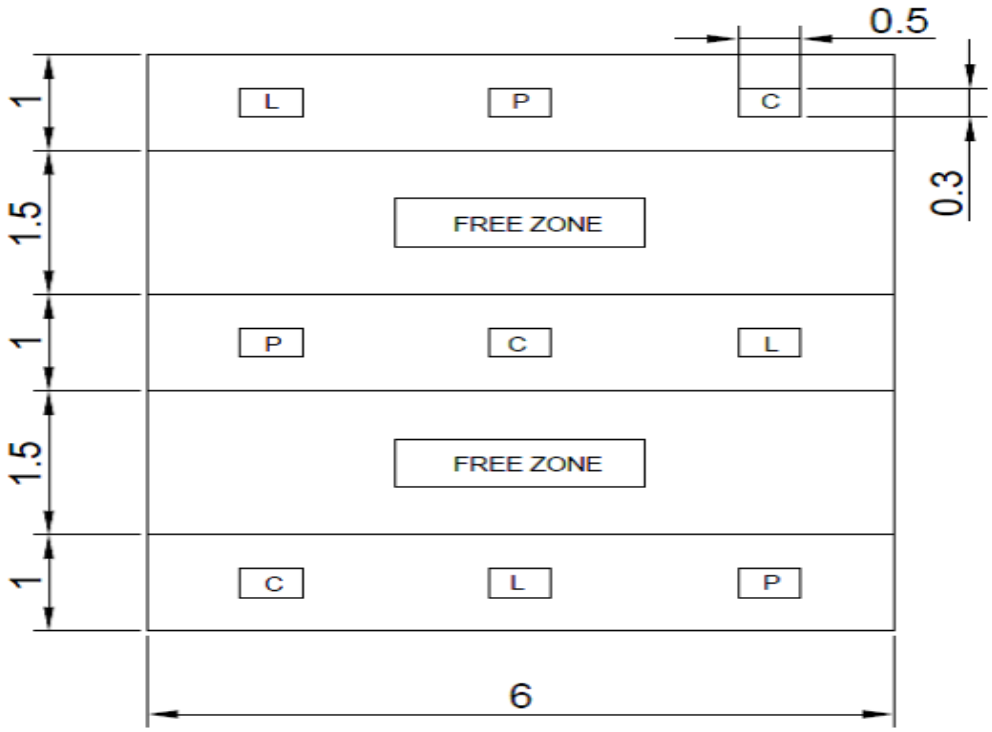


Figure 6.1: Site configuration of the control (C), leaf litter (L) and piglet (P) burials with all dimensions in metres.

The burial site (6 m by 6 m) was divided into three 1-m sections, 1.5 m apart. Each of the three 1-m sections (Figure 6.1) had three pits dug with dimensions of 50 cm (length) by 30

cm (width) by 40 cm (depth), 2 m apart for the control (C), indigenous oak leaf litter (*Quercus robur*) (L) and piglet (P).For the piglet burials, aluminium wire mesh cages (40 cm long x 25 cm wide x 15 cm high) were fabricated to prevent scavengers from gaining access. Frozen (-20°C) still-born piglets (~ 1.5 kg) were sourced from Northumbria Police (Ponteland, U.K.), transported on icepacks, re-frozen (-20°C) and thawed completely and immediately before the study burials.

Following burials, soil core samples (20-60 cm) were collected monthly from December 2014 (day 0; winter) to September 2015 (day 270; autumn) over four seasons (Table 6.1) with a gouge auger (Eijkelkamp, Netherlands) from each side of each pit. To prevent cross-contamination, the core sampler was rinsed with 99.9 % (v/v) ethanol (Thermo Fisher Scientific, Loughbrough, U.K.) after each pit sampling. The four samples from each pit were transported to the laboratory and combined prior to storage in 25 m*l* sterile universal bottles (Sarstedt, Germany). Composites of the homogenised (10 g) samples were stored (25 m*l* sterile universal bottles) at -20°C until required for both pH [2.4] and DNA extractions [2.6].

Table 6.1: Decomposition timeline by season from day 0 (December 2014) to day 270 (September 2015).

Day(s)	Month(s)	Season/Year
0 – 60	December – February	Winter 2014
90 – 150	March – May	Spring 2015
180 – 240	June – August	Summer 2015
270	September	Autumn 2015

6.2.2 Data analysis

The pH, temperature, ecological indices [2.11] and NGS [2.12] were analysed as described earlier.

6.3 Results

6.3.1 pH changes

The average pH values for the control and treatments (*S. scrofa domesticus* and leaf litter) soils were compared between day 0 (winter, December 2015) and 270 (autumn, September 2015) where , an increased pH was recorded with *S. scrofa domesticus* (8.02 ± 0.01) (Figure 6.2) on day 30 compared to control (7.86 ± 0.14) and leaf litter (7.80 ± 0.03). Subsequently, while increases in pH between days 30 and 60 were recorded for the control (8.03 ± 0.08) and leaf litter (8.05 ± 0.02), the *S. scrofa domesticus* soil decreased (7.92 ± 0.08). Both the control and leaf litter soils recorded pH decreases between days 60 and 150 (control, 7.61 ± 0.05; leaf litter, 7.70 ± 0.05) while the piglet soil showed an earlier fall between days 90 (7.96 ± 0.09) and 120 (7.66 ± 0.04) before increasing again. Likewise, both the control and leaf litter treatments then recorded pH increases between days 150 and 210. The highest pH values for the control (8.25 ± 0.14) and leaf litter (8.44 ± 0.24) were both recorded on day 210, while the highest pH value (8.02 ± 0.01) for the *S. scrofa domesticus* was recorded on day 30. All three soils recorded pH decreases between days 210 and 240 with subsequent increases to final values of 8.14 ± 0.19 (control), 8.09 ± 0.02 (leaf litter) and 7.97 ± 0.04 (piglet) on day 270. Two-way repeated measure ANOVA (RMA) showed little statistically significant temporal differences ($p = 0.064$) between the control and experimental treatments over the course of the study since only the summer period recorded a significant difference of $p = 0.039$ (Table 6.2).

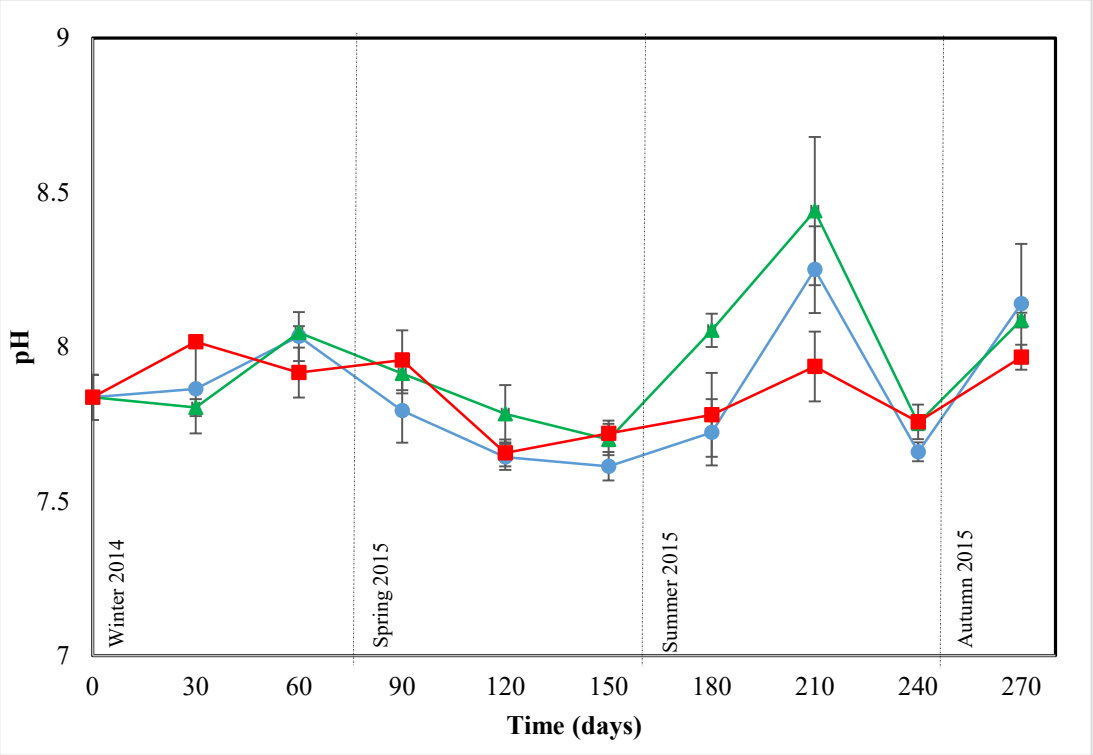


Figure 6.2: Average (n = 3) pH values of the control (●), *S. scrofa domesticus* (■) and leaf litter (▲) soils during a 270-day *in situ* study. Bars indicate standard errors.

Table 6.2: Summary of the pH ANOVA results between seasons, with significant differences ($p < 0.05$) highlighted in bold.

Winter 2014		**Spring 2015**		**Summer 2015**		**Autumn 2015**	
F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
0.104	0.902	2.370	0.122	**3.923**	**0.039**	0.600	0.579

6.3.2 Temperature

The average temperatures for the control, piglet and leaf litter burials were recorded on each sampling day (Figure 6.3) and expressed further as accumulated degree days (ADD) (Table 6.3). Decreases were recorded from day 0 (ADD 12.3) for the control (12.2 ± 0.09), *S. scrofa domesticus* (12.3 ± 0.12) and leaf litter (12.4 ± 0.19) soils to day 60 (ADD 243.3: control, 2.9 ± 0.28; *S. scrofa domesticus*, 2.9 ± 0.27; leaf litter, 2.9 ± 0.17). Seasonal change from late winter (March 2014) to summer (July 2015) resulted in temperature increases from day 90 (ADD 422.8) to day 210 (ADD 1770.7) for the control (6.5 ± 0.15; 21.7 ± 0.78), *S. scrofa domesticus* (6.3 ± 0.03; 22.1 ± 0.65) and leaf litter (6.2 ± 0.17; 21.9 ± 0.32) soils while falls,

due to seasonal weather change from late summer (August 2015) to early autumn (September 2015), were observed between days 240 (ADD 2232) and 270 (ADD 2723) for the control (17.8 ± 0.47; 15.4 ± 0.56), *S. scrofa domesticus* (17.2 ± 0.47; 14.8 ± 0.18) and leaf litter (18.5 ± 0.55; 15.4 ± 0.34) soils. Since similar trends were observed for the three soils during the study, no seasonal statistically differences were recorded (Table 6.4) and two-way RMA showed no statistically significant temporal differences ($p = 0.085$) between the control and experimental soils.

Table 6.3: Decomposition temperature timeline as expressed by ADD.

Season	Day	Control	Leaf litter	Piglet
Winter 2014	0	12.2	12.4	12.3
	30	170.5	170.2	170.3
	60	243.4	243.1	243.2
Spring 2015	90	423.0	422.3	422.5
	120	718.3	718.1	718.0
	150	902.8	903.4	902.7
Summer 2015	180	1315.6	1316.9	1314.9
	210	1770.3	1771.9	1770.0
	240	2259.1	2261.4	2258.2
Autumn 2015	270	2753.5	2755.8	2752.0

Table 6.4: Summary of the temperature ANOVA results between seasons, with no significant differences ($p < 0.05$).

Winter 2014		**Spring 2015**		**Summer 2015**		**Autumn 2015**	
F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
0.151	0.861	0.986	0.392	1.838	0.188	0.666	0.548

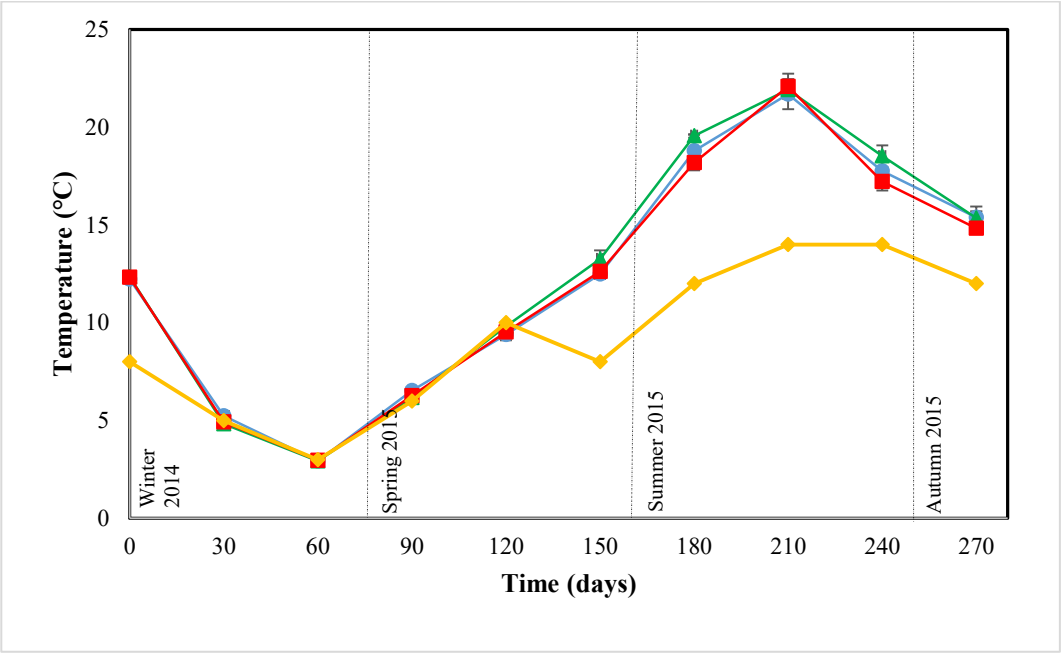


Figure 6.3: Average (n = 3) temperatures of the control (●), leaf litter (▲) and piglet (■) soils and ambient temperatures (♦) at Monk Fryston, North Yorkshire, U.K., during a 270-day *in situ* study.

6.3.3 Soil ecological analysis

As described in Chapter 2 [2.11], shifts in soil biodiversity of both 16S bacterial and 18S fungal rRNA gene profiles were analysed for richness, Shannon-Wiener index and Simpson index.

Richness

For the 16S bacterial rRNA gene richness profiles, differences were observed in the control and two treatments (Figure 6.4a). An increase in richness was recorded between days 0 (18 ± 0.6) and 30 for the control (21.3 ± 0.9) but not the piglet (19.7 ± 1.2) soil. For the leaf litter, an increase between day 60 (16.0 ± 2.5) and day 90 (24.0 ± 1.7) was followed by a decrease to 17.3 ± 1.3 on day 120. For all three soils, decreases resulted between days 180 and 240 (14.0 ± 1, control; 13.7 ± 0.9, piglet; 14.7 ± 0.7, leaf litter) and were followed by increases between days 240 and 270 (21.3 ± 1.8, control; 23.7 ± 0.3, piglet; 27.7 ± 1.9, leaf litter). The highest richness values for the control (25.0 ± 1.2) and piglet (24.7 ± 2.0) were recorded on day 180, and on day 270 for the leaf litter (27.7 ± 1.9). The two-way RMA showed no statistically significant differences (p = 0.45). Likewise, no seasonal differences

were recorded during the study (Table 6.5) although *post hoc* analysis with Tukey (HSD) identified a significant difference ($p < 0.05$) between the control and piglet soils on day 210.

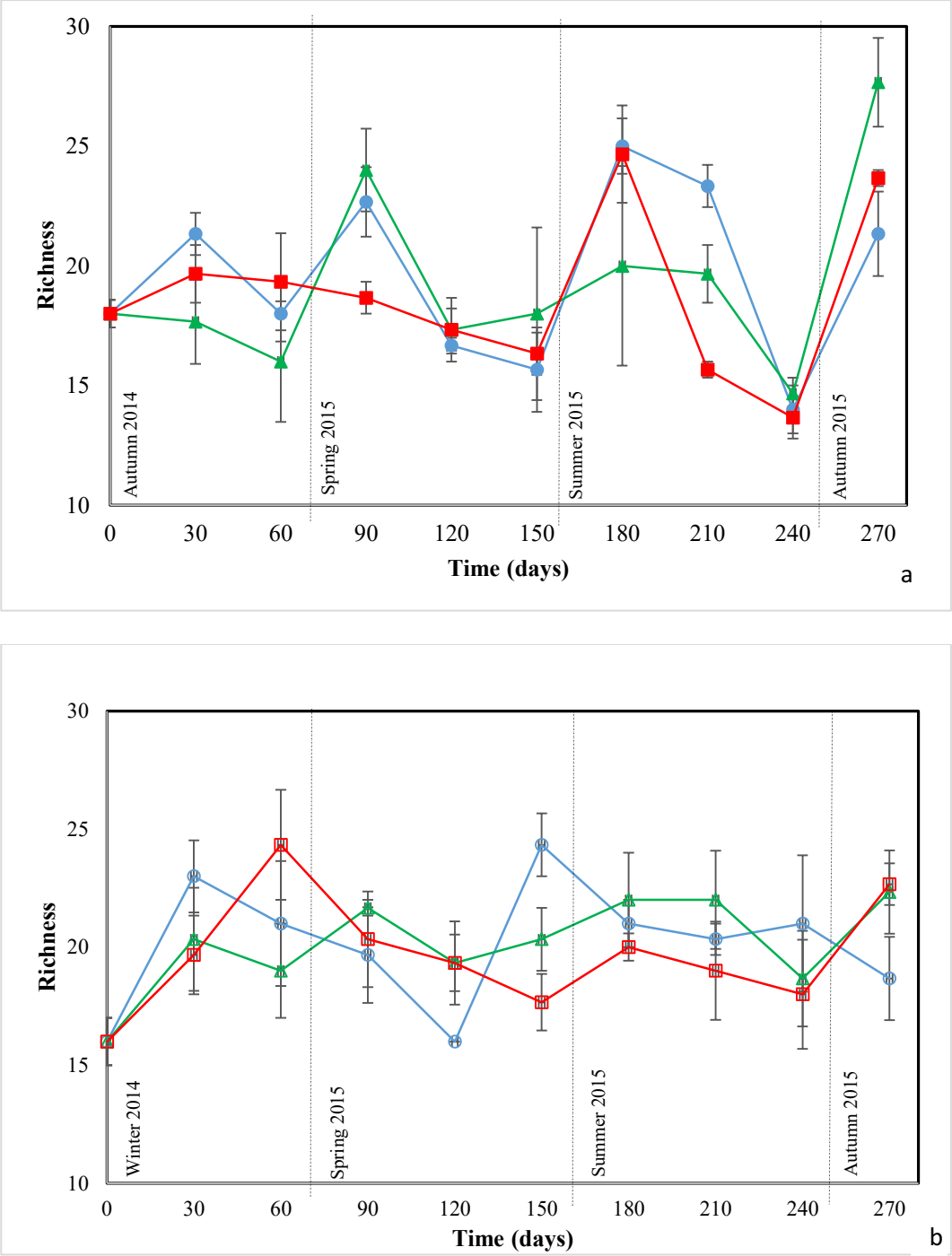


Figure 6.4: Average (n = 3) richness of 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) communities of the control (●), leaf litter (▲) and piglet (■) soils during a 270-day *in situ* study. Bars denote standard errors.

Table 6.5: Summary of the 16S and 18S richness ANOVA results between seasons, with no significant differences ($p < 0.05$).

	Winter 2014		**Spring 2015**		**Summer 2015**		**Autumn 2015**	
	F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
16S	1.675	0.215	1.491	0.252	2.443	0.115	4.617	0.061
18S	0.742	0.490	0.696	0.512	0.941	0.408	2.111	0.202

For the 18S fungal richness profiles, differences between the control and the treatment soils were observed (Figure 6.4b). For the piglet, an increase in richness up to 24.3 ± 2.3 was observed on day 60, which was followed by a progressive decrease to 18.0 ± 2.3 on day 240. Unlike the piglet, the control and leaf litter soils only recorded increases (23 ± 1.5, control; 20.3 ± 2.2, leaf litter) in winter on day 30. A decrease in richness from day 60 (21 ± 2.6) to 120 (16) was recorded for the control with further decreases between days 150 (24.3 ± 1.3) and 270 (18.7 ± 1.8). The highest richness value for the control (24.3 ± 1.3) was recorded on day 150, while those of the piglet (24.3 ± 2.3) and leaf litter (22.3 ± 1.8) were found on days 60 and 270, respectively. The two-way RMA showed no statistically significant differences ($p = 0.80$). Likewise, no seasonal differences were recorded during the study (Table 6.5) although the Tukey (HSD) *post hoc* test identified significant difference ($p < 0.05$) between the control and piglet on day 150.

Shannon-Wiener diversity

The 16S bacterial Shannon-Wiener indices showed differences between the control and experimental soils with the divergences more pronounced between days 180 (control, 2.96 ± 0.06; piglet, 2.95 ± 0.09; leaf litter, 2.69 ± 0.12) and 270 (control, 2.84 ± 0.13; piglet, 2.99 ± 0.02; leaf litter, 3.16 ± 0.09) (Figure 6.5a). While two-way RMA showed no statistically significant temporal differences ($p = 0.89$) between the control and treatments, the summer period (Table 6.6) recorded a significant difference of $p = 0.014$. Also, the Tukey (HSD)

post hoc test identified a significant difference ($p < 0.05$) between the control, leaf litter and piglet soils on day 210.

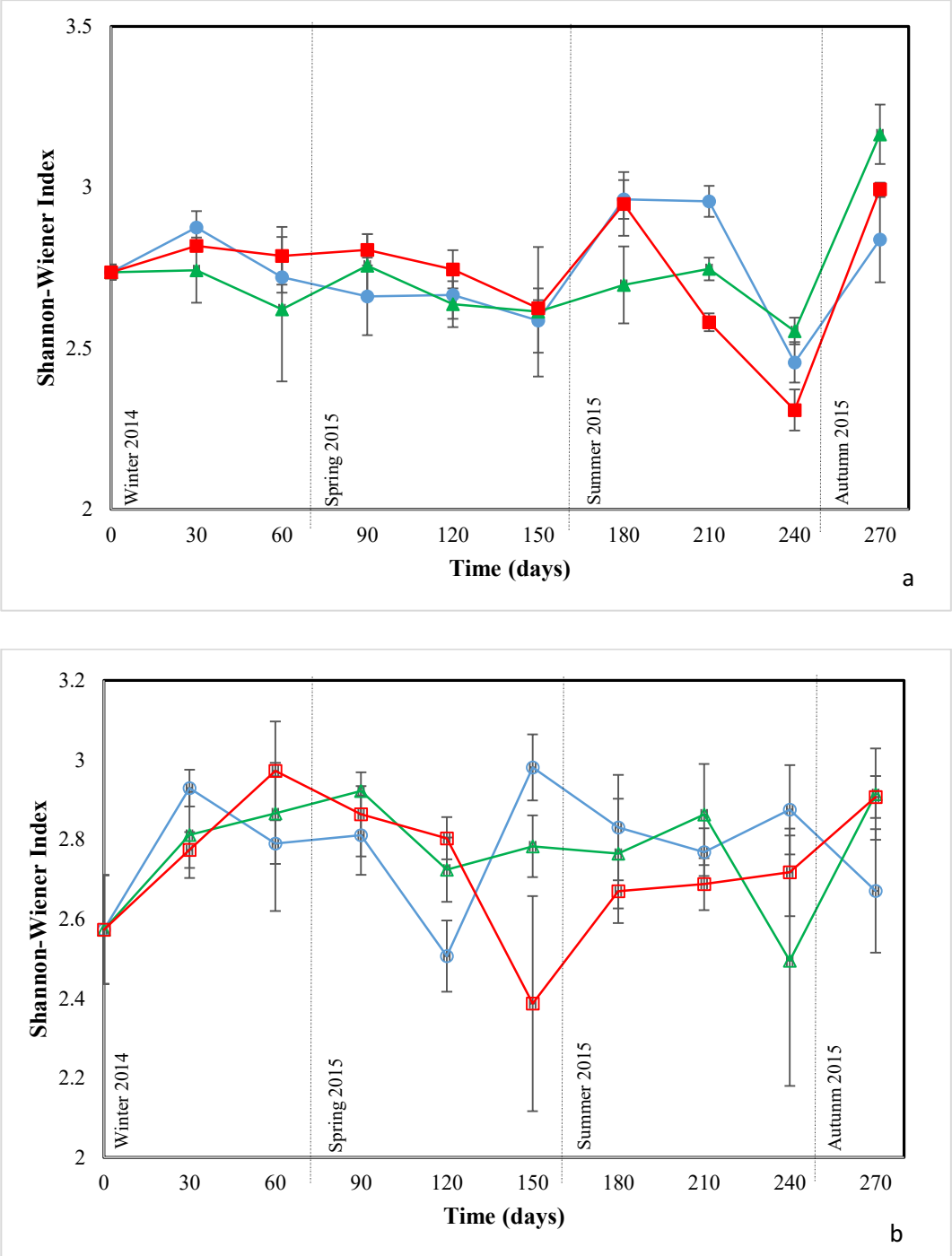


Figure 6.5: Average (n = 3) 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) communities Shannon-Wiener indices of the control (●), leaf litter (▲) and piglet (■) soils during a 270-day *in situ* study. Bars denote standard errors.

Table 6.6: Summary of the 16S and 18S Shannon-Wiener index ANOVA results between seasons, with significant differences ($p < 0.05$) highlighted in bold.

	Winter 2014		**Spring 2015**		**Summer 2015**		**Autumn 2015**	
	F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
16S	0.742	0.490	0.585	0.568	**5.498**	**0.014**	3.033	0.123
18S	0.027	0.973	0.874	0.434	0.745	0.489	1.436	0.309

The 18S fungal profiles (Figure 6.5b) showed apparent divergences for the piglet soil with increases in the Shannon-Wiener index between days 0 (2.57 ± 0.14) and 60 (2.97 ± 0.12). Further profile shifts were then observed with decreases between days 90 (2.86 ± 0.11) and 150 (2.39 ± 0.27) before increases from day 180 (2.67 ± 0.08) to day 270 (2.91± 0.05). For the control, the divergence was more pronounced with decreases between days 30 (2.93 ± 0.04) and 120 (2.51 ± 0.09) and between days 150 (2.98 ± 0.08) and 270 (2.67 ± 0.16). The leaf litter soil recorded increased divergence between days 0 (2.57 ± 0.14) and 90 (2.92 ± 0.01) with further divergence observed between days 210 (2.86 ± 0.13) and 240 (2.49 ± 0.31). Nevertheless, the two-way RMA showed no statistically significant differences (p = 0.75) due to changes in season (Table 6.6) while the *post hoc* analysis with Tukey (HSD) identified no mathematical significant differences between the control and treatment soils.

Simpson diversity

The Simpson diversity index changes, showed no statistically significant temporal differences (p = 0.86) for the 16S bacterial communities between the control and experimental soils. Decreases were, however, more pronounced between days 30 (control, 0.94 ± 0.004; leaf litter, 0.93 ± 0.01) and 90 (control, 0.89 ± 0.02; leaf litter, 0.91 ± 0.02) while increases were apparent between days 150 (control, 0.92 ± 0.01; leaf litter, 0.91 ± 0.02) and 210 (control, 0.94 ± 0.004; leaf litter, 0.93 ± 0.001) (Figure 6.6a). The piglet soil recorded a pronounced divergence with a decrease between days 180 (0.93 ± 0.01) and 240 (0.88 ± 0.01). For all the treatment soils, increases were recorded from day 240 (piglet, 0.88 ± 0.01; leaf litter, 0.92 ± 0.004) to day 270 (piglet, 0.94 ± 0.002; leaf litter, 0.95 ± 0.01). A

significant difference of $p = 0.009$ was recorded during the summer period (Table 6.7). Final analysis with the Tukey (HSD) test identified mathematically significant ($p < 0.05$) differences between the piglet and leaf litter soils on day 240.

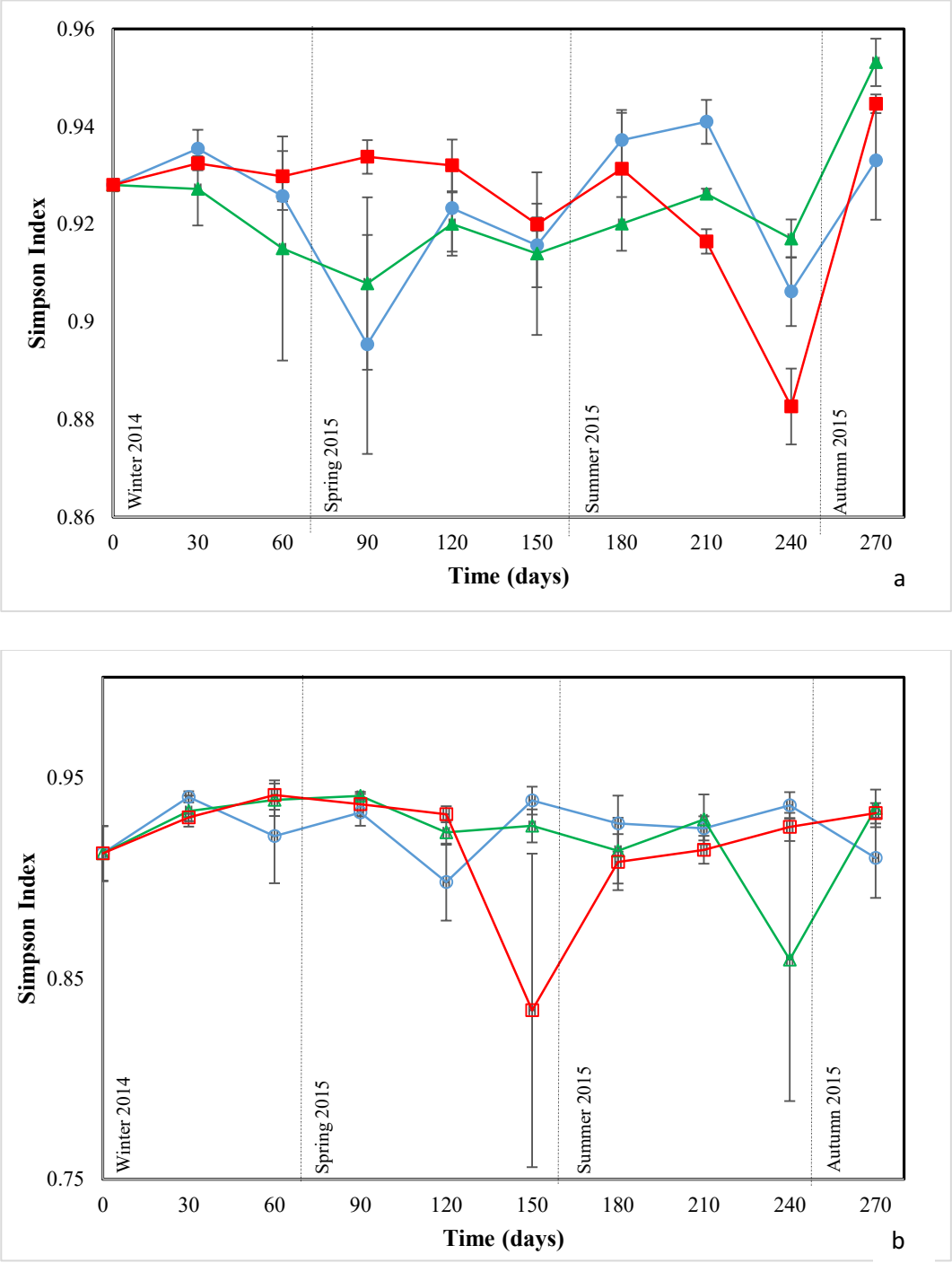


Figure 6.6: Average (n = 3) 16S bacterial (a; closed symbols) and 18S fungal (b; open symbols) communities Simpson indices of the control (●), leaf litter (▲) and piglet (■) soils during a 270-day *in situ* study. Bars denote standard errors.

The Simpson diversity index changes, showed no statistically significant temporal differences ($p = 0.75$) for the 18S fungal communities between the control and experimental soils (Figure 6.6b). Nevertheless, a decrease in diversity was recorded for the piglet soil between days 120 (0.93 ± 0.004) and 150 (0.83 ± 0.08) and the leaf litter soil between days 210 (0.93 ± 0.01) and 240 (0.86 ± 0.07). No seasonal differences were recorded during the study (Table 6.7) and *post hoc* analysis with Tukey (HSD) did not identify any significant differences ($p < 0.05$) between the control and experimental soils.

Table 6.7: Summary of the 16S and 18S Simpson index ANOVA results between seasons, with significant differences ($p < 0.05$) highlighted in bold.

	Winter 2014		**Spring 2015**		**Summer 2015**		**Autumn 2015**	
	F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
16S	0.594	0.563	1.749	0.202	**6.282**	**0.009**	1.741	0.253
18S	0.089	0.915	0.921	0.416	0.930	0.413	1.075	0.399

Principal component analysis

The 16S bacterial gene PCA biplot of the ecological measures (Figure 6.7a) showed that PC1 accounted for 62.13% of the variations in the ecological measures while PC2 accounted for 14.98%. Clustering of ecological measures for the control and treatments along the right axis showed that they were highly correlated with high measures of diversity at the sampling times. For the 18S fungal ecological measures (Figure 6.7b), PC1 accounted for 40.29% of the variations while PC2 accounted for 35.99%. In contrast to 16S bacterial ecological measures, the 18S control ecological measures showed a negative correlation to the treatments ecological measures.

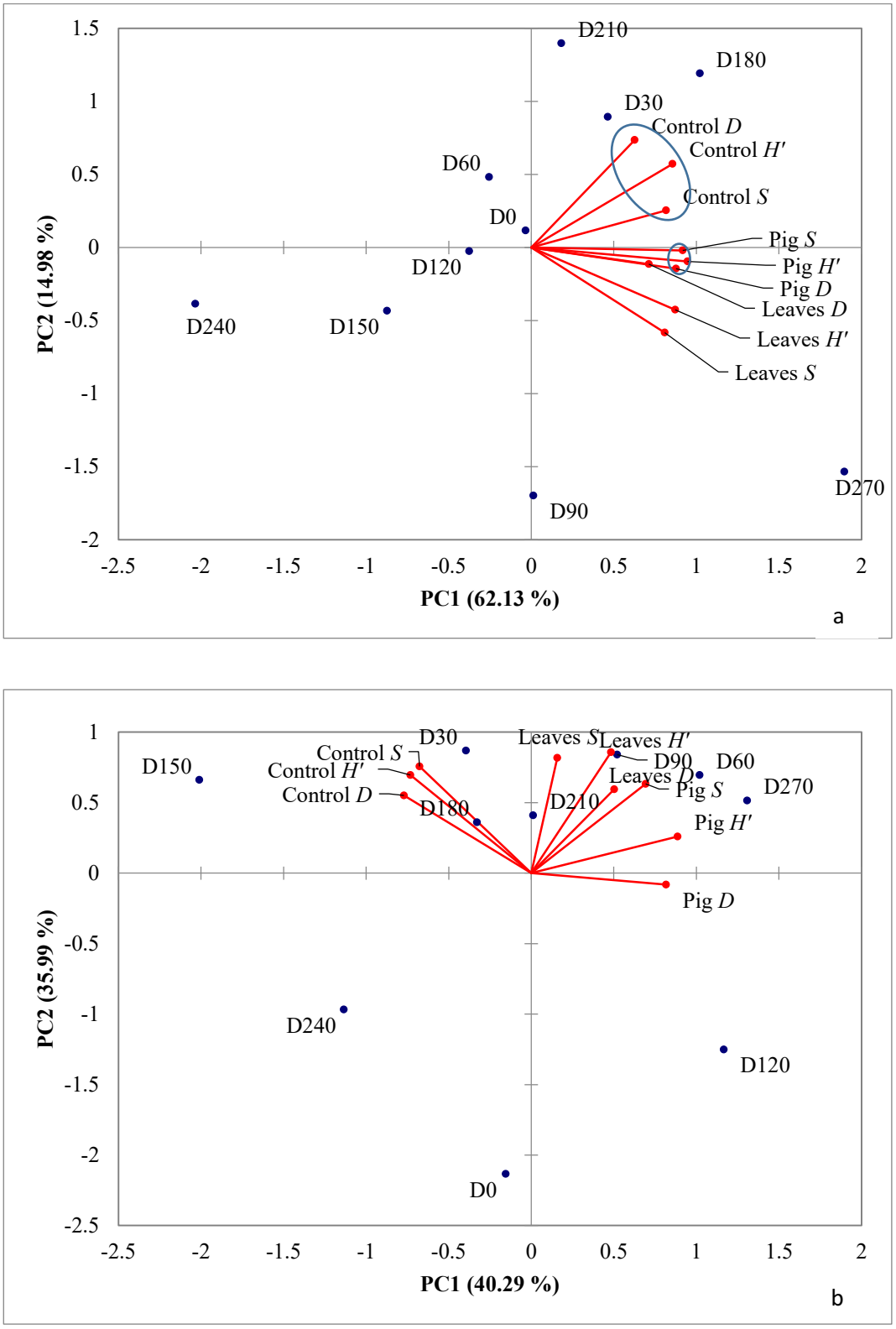


Figure 6.7: 16S bacterial (a) and 18S fungal (b) PCA biplot for ecological measures (Shannon-Wiener, *H′*; Simpson, *D*; Richness, *S*) at specific sampling times of the control, leaf litter and piglet soils.

6.3.4 Next-generation sequencing

The samples were sequenced [5.2.2] and non-bacterial sequences (*e.g.* archaea) were discarded and reads rarified at 6 750 sequences per sample. Operation taxonomic units less than 3% were classified as rare taxa. Both the rare taxa and the unclassified OTUs were omitted from the plots.

16S bacterial community taxonomic resolution

Phylum-level resolution

The dominant phyla included Proteobacteria (28.70 – 40.85%), Acidobacteria (15.04 – 32.53%), Verrucomicrobia (4.70 – 11.10%), Bacteroidetes (6.43 – 15.92%) and Actinobacteria (8.60 – 14.66%) (Figure 6.8). The NMDS visualisations revealed seasonal shifts in the bacterial communities with clusterings of most of the control and treatment samples from day 60 to day 150 while differences were observed between days 180 and 270 (Figure 6.9). Some distinct taxa, such as Chlorobi, Chloroflexi, Gemmatimonadetes and Nitrospira, correlated positively with temperature while Actinobacteria, Bacteroidetes, Planctomycetes and Verrucomicrobia correlated negatively (Table 6.8). In contrast, no taxa correlations with pH were recorded. PERMANOVA analysis showed no significant differences ($p = 0.97$) between the control and treatment soils. Similarly, the alpha diversity measures as expressed by Shannon-Wiener index box plot showed no statistically significant differences ($p = 0.41$) between the control and treatments (Figure 6.10).

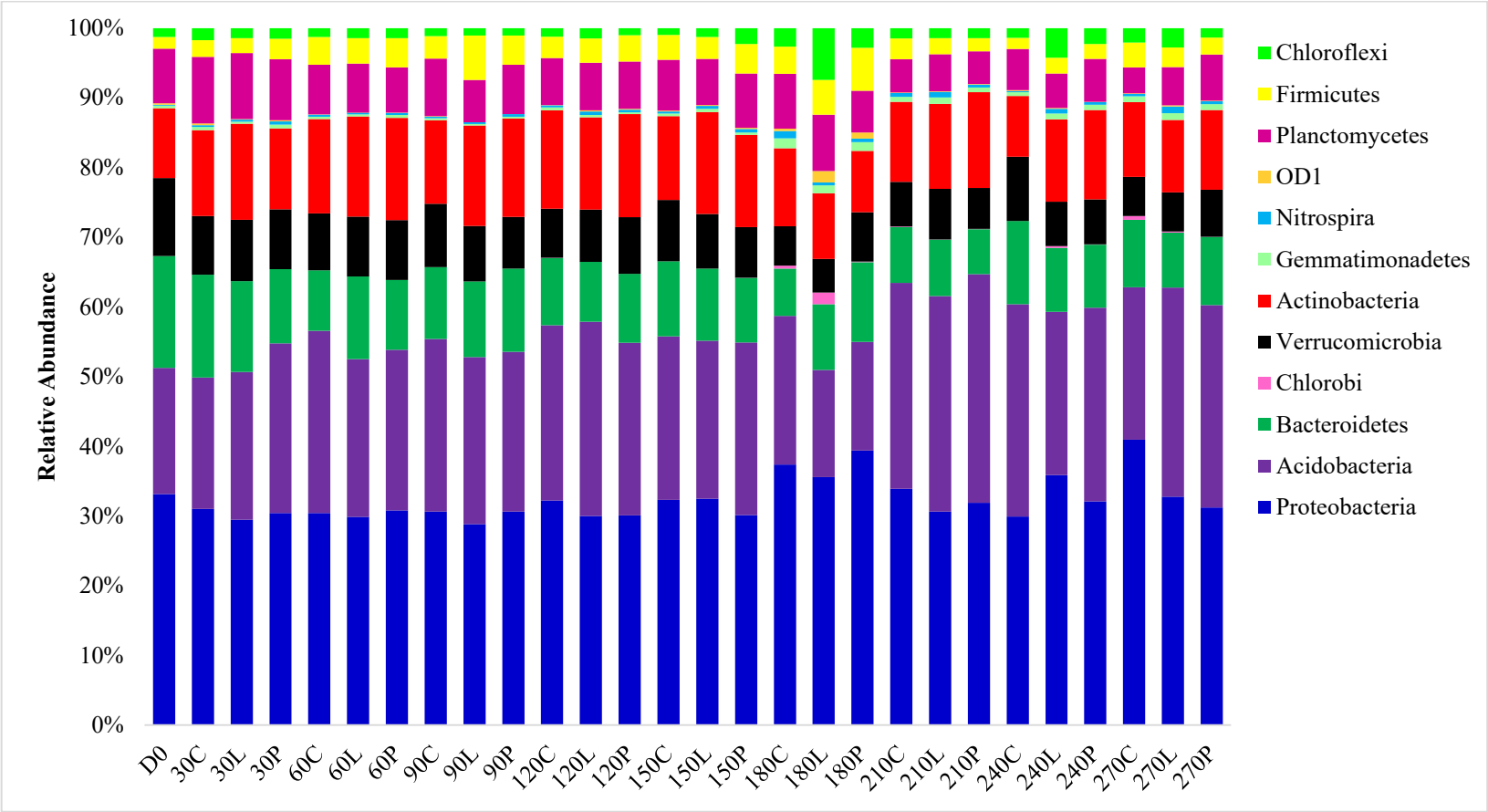


Figure 6.8: Average (n = 3) phylum-level 16S bacterial resolution of the control (C), leaf litter (L) and piglet (P) soils.

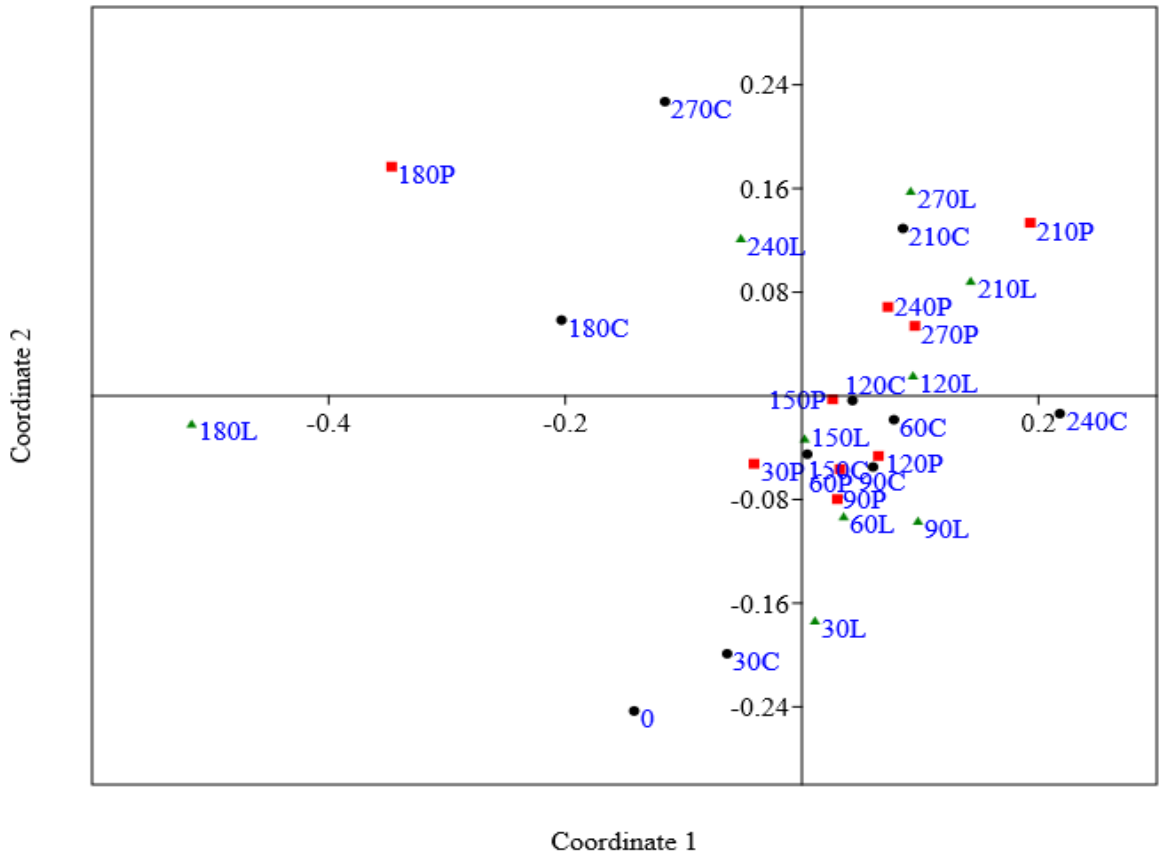


Figure 6.9: Phylum-level NMDS plot (stress = 0.11) of the control (C, ●), leaf litter (L, ▲) and piglet (P, ■) soils.

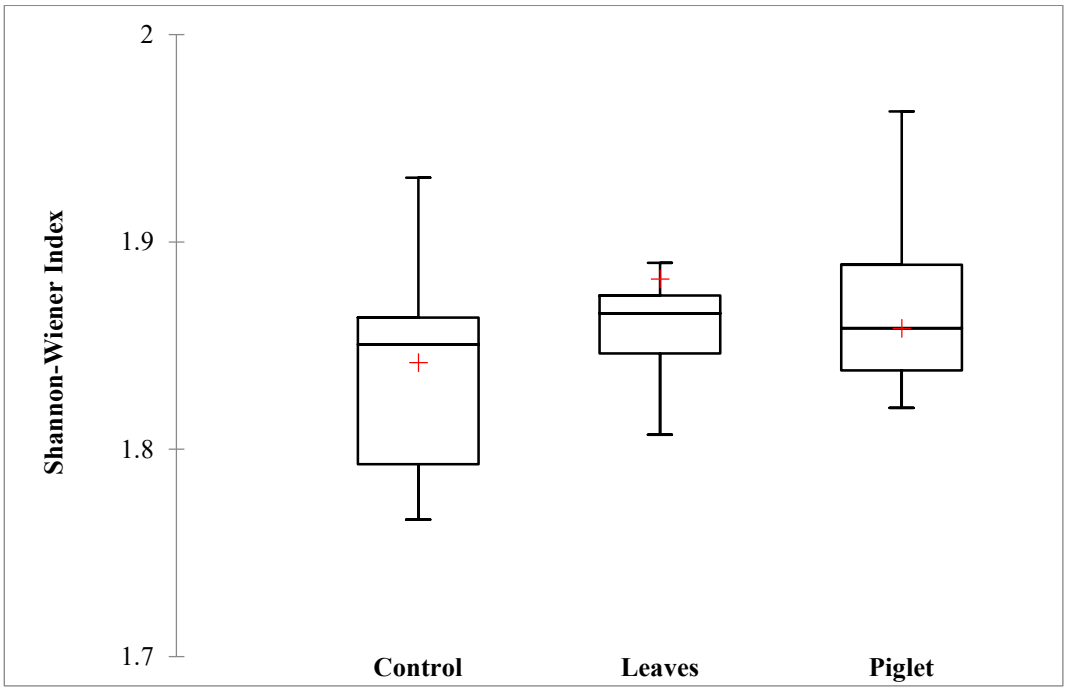


Figure 6.10: 16S bacterial taxa alpha Shannon–Wiener diversity box plot of the control, leaf litter and piglet soils.

Table 6.8: Phylum-level OTUs showing statistically significant correlations ($p < 0.05$) with temperature.

Positive			Negative		
OTUs (phylum)	**R**	***p***	**OTUs (phylum)**	**R**	***p***
Chlorobi	0.729	<0.0001	Actinobacteria	-0.563	0.002
Chloroflexi	0.422	0.026	Bacteroidetes	-0.583	0.001
Gemmatimonadetes	0.748	<0.0001	Planctomycetes	-0.673	0.0001
Nitrospira	0.573	0.002	Verrucomicrobia	-0.697	<0.0001

Order-level resolution

Taxa resolution at the order level (Figure 6.11) revealed temporal taxonomic changes of the control and treatment soils between days 180 and 270. Taxa similarities, such as Acidobacteria_Gp6_order (15.37 – 23.23%), Planctomycetales (7.55 – 12.57%), Subdivision3_order (2.42 – 7.66%), Rhizobiales (6.50 – 8.62%), Sphigobacteriales (3.93 – 7.33%) and Actinomycetales (6.06 – 8.54%), were identified between days 0 and 150, which corresponded with the winter to spring period. A shift in taxa structure was recorded on day 180 with an abundance decrease of the Acidobacteria_Gp6_order between the control and treatments, with Planctomycetales the predominant taxon of the latter. Also, increases in the relative abundances of Anaerolineales (5.58%) and Acidobacteria_Gp7_order (3.15%) were observed for the leaf litter soil. In contrast, the piglet soil recorded relative abundance increases of Methylophilales (4.01%), Methylococcales (4.97%) and Flavobacteriales (3.85%).

For all samples, shifts in taxa dominances were recorded on day 210, with increases in Acidobacteria_Gp6_order (most dominant) and Acidobacteria_Gp16_order, together with a decrease in Planctomycetales. Increases in the relative abundances of Methylococcales (3.47%) and Anaerolineales (2.63%) were

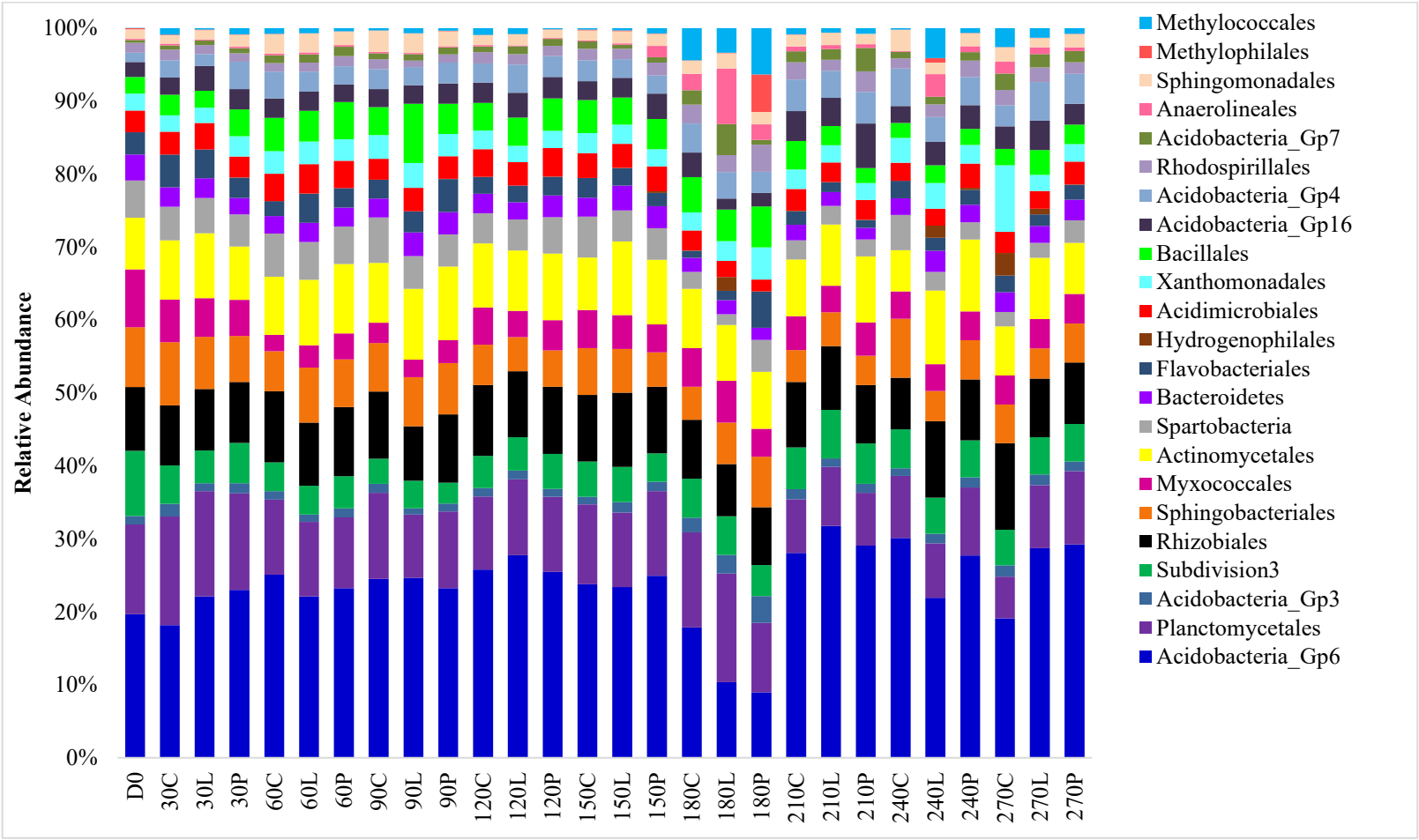


Figure 6.11: Average (n = 3) order-level 16S bacterial resolution of the control (C), leaf litter (L) and piglet (P) soils.

observed on day 240 for the leaf litter soil. For the control, further taxa changes were recorded on day 270 with an increase in the relative abundance of Xanthomonadales (7.50%) and a decrease of Acidobacteria_Gp6_order (15.72%).

Family-level resolution

A heatmap (Figure 6.12) further visualised and identified key microbial community taxa and showed distinct changes from day 180 (summer). Specifically, unique taxa such as Acidobacteria_Gp7, Anaerolineaceae, Methylococcaceae, OD1_incertae_sedis and Ignavibacteriaceae were recorded for the leaf litter while Flavobacteriaceae, Methylophilaceae and Xanthomonadaceae resulted for the piglet treatment. Taxa shifts, with increases in the relative abundances of the Acidobacteria_Gp4, Gp16_family and Subdivision3, were recorded on day 210 for all soils. On day 240, the dominance of unique taxa such as Anaerolineaceae, Methylococcaceae and Xanthomonadaceae, aerobic Gram-negative straight-rod obligate bacteria (Brenner *et al*., 2009), identified them as possible temporal indicators for the leaf litter soil. This contrasted the control where Sphingomonadaceae, Gram-negative asporogenous rods that are either aerobic or facultative anaerobic bacteria (Glaeser and Kampfer, 2014), and Flavobacteriaceae were observed. On day 270, the presence of Hydrogenophilaceae, Methylococcaceae, Xanthomonadaceae and Acidobacteria_Gp7 were recorded as summer indicators for the control.

Pairwise comparison with Turkey *post hoc* identified OTUs with statistically significant differences at family-level resolution between the control and treatment soils (Table 6.9), and relative to season (Table 6.10). For example, Hydrogenophilaceae recorded a significant difference ($p < 0.0001$) between the piglet, control and leaf litter whereas Lactobacillaceae and Xanthomonadaceae were observed to be significantly different ($p < 0.0001$) between the control and treatments. Likewise, significant seasonal differences ($p < 0.05$) were observed with OTUs such as Hydrogenophilaceae, Lactobacillaceae and

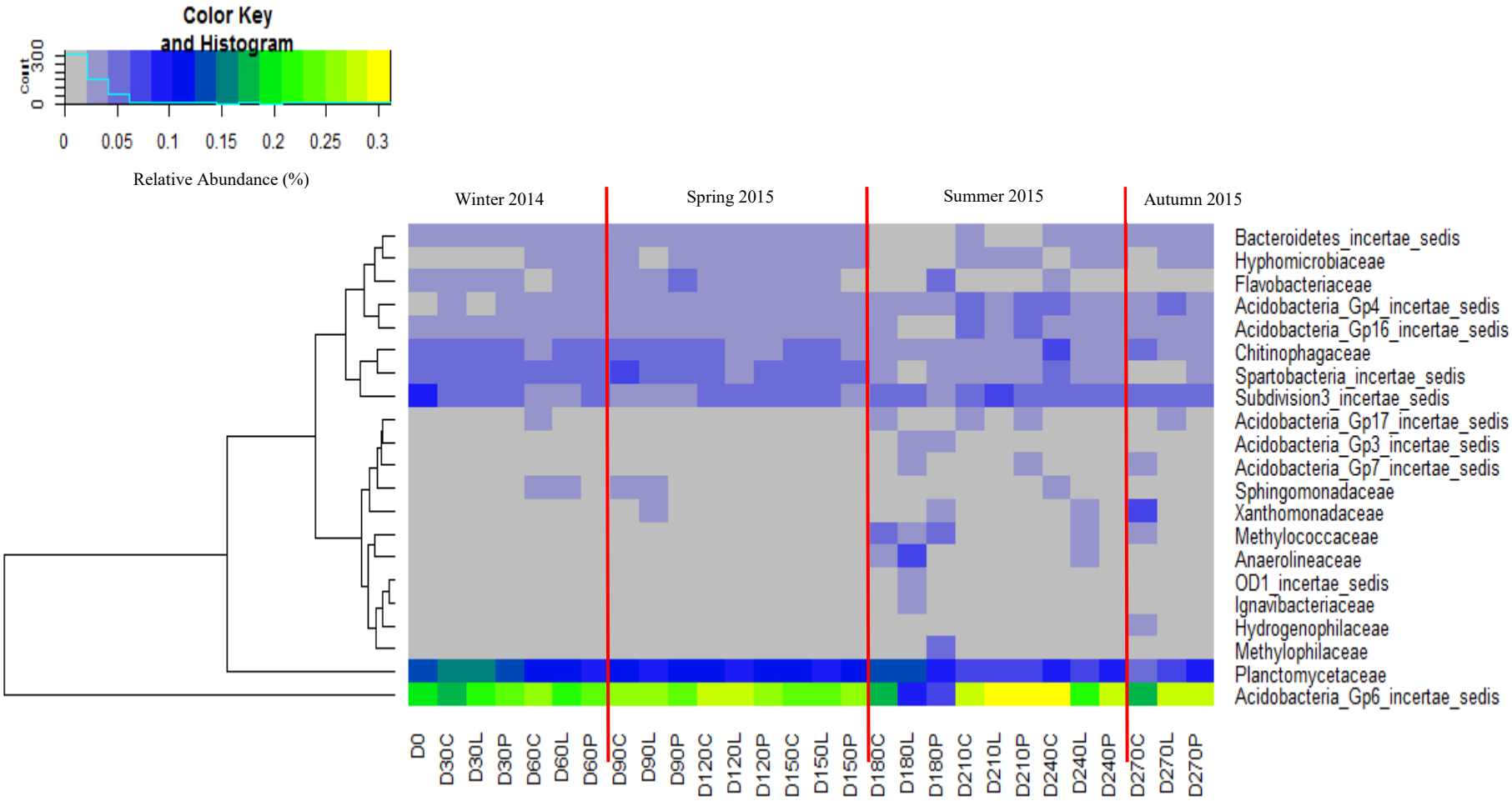


Figure 6.12: Heatmap to visualise the relative abundances (%) of the most predominant bacterial families (>0.3%) for the control (C), leaf litter (L) and piglet (P) soils.

Xanthomonadaceae observed to differentiate autumn 2015 ($p < 0.0001$) while Planctomycetaceae and Spartobacteria_incertae recorded significant differences ($p < 0.0001$) between winter 2014, and spring, summer and autumn 2015.

Table 6.9: Family-level OTUs that were significantly different between the control and treatments soils according to the least squares means (LS-means). Combinations sharing the same letter (a, b) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTUs (family)	Control	Pig	Leaf litter	*p*
Hydrogenophilaceae	27.47 a	2.31 b	15.25 a	0.000
Lactobacillaceae	21.03 a	0.50 b	0.17 b	0.000
Xanthomonadaceae	105.53 a	46.89 b	46.08 b	0.000

Table 6.10: Family-level OTUs that were statistically significantly different between seasons according to LS-means. Combinations sharing the same letter (a, b) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) *post hoc* tests.

OTUs (family)	Winter 2014	Spring 2015	Summer 2015	Autumn 2015	*p*
Hydrogenophilaceae	0.33 b	1.26 b	13.56 b	44.89 a	0.000
Lactobacillaceae	0.00 b	0.22 b	0.70 b	28.00 a	0.000
Planctomycetaceae	411.22 a	373.11 a	297.70b	272.78 b	0.004
Spartobacteria_incertae	172.00 a	169.19 a	93.11 b	80.00 b	0.003
Xanthomonadaceae	48.22 b	47.82 b	54.85b	113.78 a	0.000

6.4 Discussion

pH and temperature

An increase in the *Sus scrofa domesticus* treatment pH between days 0 and 30 was in agreement with earlier reports where increases were attributed to the: release of ammonia-rich fluid; mineralisation of base-forming cations (Ca^{2+}, K^{+} and Mg^{2+}); and ammonification of organic nitrogen (protein and peptide) (Hopkins *et al.*, 2000; Meyer *et al.*, 2013).

Similarly, the subsequent pH decrease was supported by the work of Meyer *et al*. (2013) who suggested that nitrate accumulation was responsible. The pH differences recorded for the *Sus scrofa domesticus* and *Quercus robur* leaf litter soils can possibly be related to their nutrient contents (Tagliavini *et al*., 2007; Janaway *et al*., 2009) and decomposition rates, which have been reported to be slower for plants (Metcalf *et al*., 2016). Such slowing was more evident during the summer period when a significant difference (p = 0.039) was recorded in conjunction with the highest pH value of 8.44 for the leaf litter after 210 days.

Microbial activities are influenced by ambient temperature with higher activity recorded between 21 and 38°C and a lower activity resulting below 4°C (Stokes *et al*., 2009). During the study, an ambient temperature decrease occurred from day 0 (8°C) to day 60 (3°C) (autumn 2014) while a seasonal change from spring to summer (days 90 to 210) corresponded to an increase to 14°C before the change from summer to early autumn (days 240 to 270) resulted in a decrease to 12°C. While it might be difficult to classify accurately the decomposition stages in this particular study, the seasonal changes were used subsequently to consider the taxa differences along the decomposition timeline. Specifically, Chlorobi, anaerobic phototrophic green sulphur bacteria (Imhoff 2014), Chloroflexi, Gram-negative filamentous bacteria (Hanada 2014), Gemmatimonadetes, Gram-negative mesophilic aerobic bacteria (Hanada and Sekiguchi, 2014) and Nitrospira, Gram-negative chemolithoautotrophic aerobic nitrite-oxidising bacteria (Daims 2014), have all been reported to proliferate between 15 and 45°C. This was apparent as positive correlations of these taxa with temperature with increases in their relative abundances between summer 2015 and autumn 2015.

All future work should include complementary analyses of specific environmental variables and physicochemical analysis, including key nutrient profiling (*e.g.* Tibbett *et al*., 2004; Benninger *et al.,* 2008; Damann *et al*., 2012), parallel to microbiome dynamics (*e.g.* Cobaugh *et al*., 2015).

DGGE-based ecological indices

Even though the ecological measures of richness, Shannon-Wiener index and Simpson index did not show statistical differences for the overall decomposition timeline, visual inspection suggested that there were differences between the control and the treatments. Further to this, mathematical analyses at specific sampling times revealed some statistical differences. For example, the 16S bacteria results for the control were visually different from those of both the piglet and leaf litter treatments and *post hoc* analysis with Tukey (HSD) identified: significant differences ($p < 0.05$) in richness between the control and piglet soils on day 210; and significant differences ($p < 0.05$) in the Shannon-Wiener diversity indices between the control, leaf litter and piglet soils on day 210, and between the piglet and leaf litter soils ($p < 0.05$) on day 240. Likewise, seasonal differences ($p < 0.05$) were recorded for the Shannon-Wiener and Simpson indices during the summer period, which aligned with the earlier results of the *post hoc* analysis with Tukey (HSD). The 18S changes also revealed visual differences between the control and treatments although only richness revealed significant difference ($p < 0.05$) by *post hoc* analysis between the control and piglet on day 150. No seasonal differences were recorded for the 18S changes.

Ecological measures of diversity have often reflected species richness and relative abundance of soil communities so it is important to use more than one ecological measure to avoid potential underestimations of community biodiversity (Bandeira *et al.*, 2013). Although caution should be exercised when using DGGE due to some of its limitations, the results of this study were useful as they differentiated between the control and treatment soils for the summer.

As indicated in Chapter 1, multivariate analyses are being applied increasingly in forensic studies that are underpinned by microbial ecology techniques since they facilitate comparisons of multiple data sets from the same investigation. By their visual clustering, for the current *in situ* study, the ecological measures (richness, Shannon-Wiener index and

Simpson index) for both the control and leaf litter soils appeared more closely correlated than the piglet soil. Nevertheless, the measures were highly correlated over the total decomposition timeline since evaluation of the 16S bacterial and 18S fungal PC1 and PC2 by ANOVA showed no significant differences ($p < 0.05$).

16S bacterial community taxonomic resolution

The potential use of epinecrotic communities as the "postmortem microbial clock" (Metcalf *et al*., 2013; Finley *et al*., 2015b) highlighted the need for more comprehensive decomposition studies. While characteristically predominant in soils (Peralta *et al*., 2013; Ligi *et al*., 2014; Arroyo *et al*., 2015), the numerical abundances of Proteobacteria, Acidobacteria and Actinobacteria specifically in decomposition-impacted soils have been reported by various researchers (Carter *et al*., 2015; Hoppe *et al*., 2015; Finley *et al*., 2016). As observed in this study, Proteobacteria was found to be the most abundant phylum throughout. The abundances of Acidobacteria, oligotrophic bacteria found in soil, have been reported to correlate with soil pH (Jones *et al*., 2009; Lauber *et al*., 2009) although this was not apparent in the study or in the studies of both Metcalf *et al*. (2013) and Cobaugh *et al*. (2015). Seasonal changes from spring to summer resulted in increases in some taxa relative abundances as was noticeable with the positive correlations of Chlorobi, Chloroflexi, Gemmatimonadetes and Nitrospira from day 180/summer period. Overall, phylum-level differentiation between the control and treatment soils was apparent in the summer months with Proteobacteria as the seasonal community structure-based PMI and time-since-burial indicator.

Order-level resolution matched the phylum-based profiling where microbial community structure shifts were seemingly season-dependent as reflected between early summer (day 180; June, 2015) and early autumn (day 270; September, 2015) when ADD maxima between 1314.9 and 2755.8 were also recorded. These were evidenced by increased abundances of Methylophilales, Methylococcales and Flavobacteriales for the mammalian surrogate

treatment. Specifically, the Methylophilales recorded considerably low relative abundances (<2%) at most sampling times. Therefore, their differential presence and absence identified this as a probable order-level "microbial clock" indicator relative to community composition.

Generally, the abundances of Methylophilales and Methylococcales confirmed anaerobic conditions *in situ* and the sensitivities of the rare methylotrophic denitrifies and methanogenic orders, respectively, as biomarkers for decomposition. Notwithstanding this, the increased abundances of Methylococcales in the control and treatment soils on day 180, and on day 240 in the presence of leaf litter, indicated its possible limitation as a differentiating taxon between *S. scrofa domesticus* and leaf matter decomposition. The increased abundance of the order Flavobacteriales suggested a piglet decomposition-mediated enrichment of commensal opportunistic pathogenic strains that are typically found in marine (animals) and contaminated or waste treatment ecosystems (Daley *et al*., 2016; Qiu *et al*., 2016). Therefore, analysis of the indigenous pig necrobiome parallel to the burial soil microbiome must be considered in future research work.

The control and treatments phyla were comparable between winter (day 0, December 2014) and spring (day 150, May 2015) while the family-level resolutions (Figure 6.12) revealed greater phylogenetic variations with pronounced taxa community shifts recorded during the summer season (days 180 to 270).

Increases in Methylococcaceae, aerobic Gram-negative methane-oxidising bacteria (Brenner *et al*., 2009), and decreases in Acidobacteria_Gp6_family were recorded in all soils on day 180. The predominance of obligate anaerobes, such as Anaerolineaceae, which are associated with anaerobic degradation of crude oil related compounds (Liang *et al*., 2015), Gram-negative rod-shaped non-sporulating anaerobic Ignavibacteriaceae (Iino, 2014) and Gram-negative sulphur-oxidising Hydrogenophilaceae (Brenner *et al*., 2005) were recorded for the leaf litter soil on day 180.In contrast, aerobic Gram-negative methanol-oxidising

(Brenner *et al*., 2005) and dimethylsulphide-degrading (Eyice *et al*., 2015) Methylophilaceae (Brenner *et al*., 2005) and Flavobacteriaceae, chemoorganotropic Gram-negative aerobic bacteria (Krieg *et al*., 2010), dominated the piglet soil. The first predominances contrasted an earlier report by Purahong *et al*. (2016), where genera such as *Frigoribacterium* and *Sphingomonas*, known for their proteolytic and cellulolytic enzymes, were recorded during the early stages of leaf litter decomposition. These contrasts may be attributed to different leaf litter types – plant species and their respective cellulose, hemicellulose and lignin contents – and experimental designs (litter bags vs *in situ*).

The occurrence and numerical abundance of Methylophilaceae on day 180 (early summer) in the presence of *Sus scrofa domesticus* identified this family as a likely community composition- and structure-based "microbial clock" indicator and suggested a possible influx of methanol. Although outwith the scope of this study, methanol could be a biochemical signal for targeting in complementary forensic chemistry and forensic ecogenomic analyses. Furthermore, the roles of chemical signals, including methanol, are well recognized in forensic entomology and justify parallel adoption of this discipline to address further key knowledge gaps in forensic subsurface decomposition study.

Taxa shifts with increases in relative abundances of the Acidobacteria_Gp4, Gp7 and Gp16_family were recorded on day 210 for all soils. For the *Quercus robur* treatment, decreased Anaerolineaceae relative abundance was recorded on day 240 while increases in Hydrogenophilaceae and Xanthomonadaceae were found for the control on day 270. As reported by Purahong *et al.* (2014), microbial communities involved in litter decomposition undergo temporal season-dependent changes, which were also observed during this study. The presence of nitrogen-fixing Rhizobiales recorded for the control and treatments throughout contrasted the work of Hoppe *et al.* (2015) who reported them as mutualistic bacteria associated with fungi in *Picea abies* and *Fagus sylvatica* deadwood log decomposition and whose abundance increased in late intermediate to advanced decay

stages. Notwithstanding this, some similarities were apparent. Hoppe *et al.* (2015), for example, recorded the presence of methanotrophic bacteria in deadwood logs, with the most abundant from the *Methylovirgula* genus, while the current study recorded the presence of Methylococcaceae in both the control and treatments, and Methylophilaceae in the piglet soil.

A report by Weiss *et al*. (2016) identified marginal differences in the epinecrotic community of a 1 kg swine compared with 20 – 50 kg carcasses and recommended the use of the latter as human surrogates in postmortem microbial investigations. In the current study ~1.5 kg piglets were used, which are recognized as good models for research purposes (Benbow *et al*., 2015). Although it was assumed that the piglets had relatively high lipid contents, Charneca *et al*. (2010) reported higher protein to lipid ratios in newborn piglets. Therefore, future studies should investigate the effects of age and nutrient composition on envrionmental/soil microbial community dynamics**.**

6.5 Conclusions

Determinations of the effects of abiotic factors on cadaver decomposition rates are essential in postmortem and time-since-burial investigations. Since dead plant organic matter, or litter, is a unique and considerable energy source, its decompositional impacts on occurring soil microbial community dynamics must be explored specifically for direct comparisons with carcass-based influences within forensic ecogenomics. As observed in this study, soil temperature differences related directly to seasonal differences in decomposition activities, as expressed by taxa relative abundances, identified by next-generation sequencing. NGS revealed that microbial community shifts were more evident through the summer season (from day 180, June 2015). Also, some unique taxa, such as Methylococcaceae, could be used generally, as community structure-based seasonal indicators/predictors as observed from day 180. In particular, Anaerolineaceae, recorded for the *Quercus robur* leaf litter soil, and Methylophilaceae, especially the Methylophilales order, recorded for the piglet, could

be “postmortem microbial clock” and time-since-burial determinants for the two different substrates in the summer for burials associated with similar soils or sites.

Of the three programme hypotheses, all three were rejected at phylum-level resolution since only the abundance of specific phyla (structure) changed temporally and in response to decomposition. Specifically, a Shannon-Wiener diversity box plot revealed no statistically significant differences (p = 0.41) between the control and treatments at phylum-level taxonomic resolution.

Hypothesis 1 was accepted at family-level where increases in the relative abundances of Methylophilaceae and Flavobacteriaceae were recorded for the *S. scrofa domesticus,* while increases in the relative abundances of Chitinophagaceae, Xanthomonadaceae, Hydrogenophilaceae and Methylococcaceae were observed for the control. Hypotheses 2 and 3 were both accepted at family-level where some community composition deliniation existed between the control and leaf litter due to the presence and increased abundances of Anaerolineaceae, Ignavibacteriaceae and Hydrogenophilaceae, while Methylophilaceae and Flavobacteriaceae were observed for the piglet treatment. Also, the numerical abundances of several members such as Sphingomonadaceae, Hydrogenophilaceae and Xanthomonadaceae identified them as potential community structure-based indicators to differentiate between the control, leaf litter and piglet treatments on days 240 (late summer 2015) and 270 (early autumn 2015). In contrast to phylum-level analysis, family-level resolution could, possibly, be used to locate clandestine burials for this soil type particularly during summer to early autumn. Repeat studies over consecutive/different years are, however, required to provide conclusive evidence for core family-mediated seasonal microbial clocks.

This study was made with piglets as surrogates for human cadavers so the results, possibly, provided information relevant to suspected clandestine child burial investigations in contrast to those of adults. Since, the piglets were frozen prior to on-site thawing, the assumption

was made that the impacts of their indigenous microbiome would be minimal. Therefore, future work should expand on earlier investigations such as those of Stokes *et al*. (2009) and Cobaugh *et al.* (2015) and consider the effects of cadaver/surrogate storage, in general, and freezing, in particular. Overall, further replicated subsurface investigations at different sites/regions, and with different cadaver/proxy sizes, leaf/plant litters and soil types should resolve the potential of soil mirrobiome changes as a forensic tool.

Chapter 7: General conclusions and future work

7.1 Introduction

Cadaver decomposition is a complex process with microbial activity playing an important role. Nevertheless, the complex interactions of the cadaveric and environmental microbial communities in both surface and subsurface decompositions still remain a "black box" for forensic practitioners. Studies are beginning to address the knowledge gap *re* cadaver decomposition with a view to using microbial community profiles as potential tools in postmortem and postburial interval estimations. To address this paucity, four different studies were made in this research programme. Study I explored temporal changes in soil microbial communities during *S. scrofa domesticus* decomposition relative to burial depth while Study II compared shifts in microbial biodiversity in response to animal tissue and plant litter carbon sources with links made also to key environmental variables. Study III used whole piglets and compared changes in microbial community structure and composition in the presence and absence of two carbon sources. Finally, Study IV explored *in situ* underground decompositions of two carbon sources (piglet and leaf litter) where the environmental variables of soil pH and temperature were also monitored.

7.2 General conclusions

This research programme was designed to address a key knowledge gap by testing the overarching hypothesis that microbial ecological tools can be applied in subsurface decomposition studies for PMI estimation and identification of potential microbial clock indicators. Specifically:

1. The subsurface decomposition of tissue and/or whole *S. scrofa domesticus* as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.

2. The subsurface decomposition of tissue and/or whole *S. scrofa domesticus* will effect different shifts in the surrounding soil microbiome structure and composition when compared to plant/leaf litter.
3. Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and plant/leaf litter decompositions.

Overall, the results of these studies reflected published reports and exemplified that abiotic factors such as temperature and pH are important variables that must be considered in PMI estimations. As observed from Studies II – IV, increased pH values for the *S. scrofa domesticus* soils were recorded in the early phases, which may be indicative of the added substrate composition in comparison with plant/leaf litter as non-pig carbon sources and the controls. Also, the mixed carbon sources effected pH changes that contrasted the individual substrates, which were probably attributable to differences in soil microbial metabolic and activity rates in relation to catabolic products of the individual decomposition substrates. Understanding the carbon source type, their chemical compositions and respective metabolic/catabolic products could be useful, potentially, when considering the possibility of cadaver/plant litter mixtures in clandestine graves.

Soil temperature changes from Studies II – IV revealed that substrate catabolism and subsequent shifts in microbial community profiles can be related directly to variations in seasonal weather. Also, seasonal temperature changes, which were more pronounced during the summer periods, resulted in shifts in microbial community composition as expressed by the ecological indices of richness, Shannon-Wiener diversity and Simpson diversity, and next-generation sequencing. For example, increased taxa abundances were more pronounced during the summer periods, which are linked directly to increases in temperature. As a consequence, the impacts of changes in weather and climate on the microbiome must be studied further to assess the effective, relevant and appropriate use of postmortem microbial clock indicators in PMI estimation. Protracted future studies, in different geographical

locations, and over different/consecutive years, should then consider the long-term implications of global climate change where monthly climatic conditions vary between years, which will, invariably, affect rates of decomposition and related microbial community dynamics.

Analysis and interpretation of the PCR-DGGE fingerprints by Shannon-Wiener diversity, Simpson diversity, richness and evenness ecological indices revealed changes in the microbial community structure relative to the decomposition timelines. Key findings were that profiling of the bacterial 16S rRNA gene was seemingly more suitable for *S. scrofa domesticus* PMI estimation while ecological indices of the fungal 18S rRNA gene were better indicators of plant/leaf litter decomposition. Due to its limitations, the microbial community profiles may be underestimated by denaturing gradient gel electrophoresis and so recommend use of next-generation sequencing.

The metagenomic profiles revealed decomposition-mediated changes within the soil microbiota. This programme is one of the first comprising subsurface investigations and certainly the first research where epinecrotic communities of two carbon sources were compared in a forensic ecogenomic context. The results highlighted the potential of using these communities for PMI estimation and revealed that family-level resolutions identified detailed phylogenetic variations at longer time intervals. Some unique taxa, such as Sphingobacteriaceae and Xanthomonadaceae, provided preliminary indicators to differentiate between pig and plant litter decomposition as illustrated in Study II. Likewise, taxa such as Sphingobacteriaceae and Alcaligenaceae were identified during the summer as potential seasonal indicators for piglet and piglet + plant treatments in Study III whereas Planctomycetaceae and Sphingobacteriaceae were likely indicators to differentiate between the two carbon sources. Finally, the *in situ* epinecrotic community profiles identified Anaerolineaceae and Methylophilaceae as microbial clock indicators for leaf litter and piglet, respectively, for decomposition in Soil IV and, potentially, similar soil types. A

summary of taxa identified as likely PMI/PBI and season indicators are highlighted in Table 7.1 while a summary of the tested hypotheses, with key evidences indicating the novelty of the research programme, are highlighted in Table 7.2. The latter also identifies question from the research programme, which can be pursued possibly in future work.

7.3 Review of experimental procedures

The impacts of decomposition on different soil types is essential to investigate. Naturally, this would require the use of the same animal model regarding species, age, size or tissue type if the whole mammalian proxy is not used. Setting up the studies in this research programme had some challenges hence the use of three different soils *ex situ*. These included: (i) loss of research soil in the laboratory; (ii) absence of a body farm for extended research; and (iii) limited financial resources. Considerable thought was put into the programme design where a preliminary study would be followed with an extended 2-year *in situ* study consisting of adult pigs. Notwithstanding this, miscommunication with site owners, the absence of site approval and fear of animal rights organisations in the U.K. discouraged land owners' acceptance of research facilities on their land. To mitigate for delayed site approval, additional replicated/un-replicated microcosm studies were used and, hence, the three different soil types, skeletal muscle tissues and piglets. While these mitigation strategies may have confounded the results to some extent and especially in relation to cross-study comparability, they ensured successful progress and timely completion of the research programme to then attain its aims and address key knowledge gaps.

Table 7.1: Dominant and/or numerically abundant taxa at family-level for Study II – IV as determined by next-generation sequencing. Focus is made on taxa that were identified as likely PMI/PBI† and season‡ indicators. † Designates likely PMI/PBI indicators; ‡ designates likely season indicators.

Control				***S. scrofa domesticus* (pig)**			***Agrostis*/*Festuca* spp (Plant litter)**		**Pig + Plant**	***Quercus robur* (Oak leaves)**
	II	**III**	**IV**	**II**	**III**	**IV**	**II**	**III**	**II**	**IV**
1	Alicyclobacillaceae†‡	Hyphomicrobiaceae†‡	Hydrogenophilaceae†‡	Micrococcaceae†‡	Microbacteriaceae†‡	Flavobacteriaceae†	Xanthomonadaceae†‡	Planctomycetaceae†‡	Sphingobacteriaceae†‡	Anaerolineaceae†
2	Comamonadaceae†‡	Acidobacteria_Gp4†	Lactobacillaceae†‡	Sphingobacteriaceae†‡	Alcaligenaceae†‡	Methylophilaceae†		Comamonadaceae†‡	Porphyromonadaceae†‡	Methylococcaceae†
3		Xanthomonadaceae‡	Xanthomonadaceae†‡	Staphylococcaceae†‡	Sphingobacteriaceae†‡	Xanthomonadaceae†‡			Alcaligenaceae†‡	Ignavibacteriaceae†
4			Chitinophagaceae‡	Alcaligenaceae†‡					Xanthomonadacea†‡	Acidobacteria_Gp7†
5				Micromonosporaceae †‡					Flavobacteriaceae†	OD1_incertae_sedis†

Table 7.2: A mapping of the research programme methods, hypotheses and evidence of their achievements, the novelty from specific studies and knowledge gaps for potential future work.

Study number, design and thesis chapter	**Programme hypothesis (reworded relative to each study design)**	**Key evidence of the hypothesis**	**Questions for future work**
Study I_Chapter 3: Whole pig leg; 20 kg Soil I; Temporal 16S and 18S DGGE profiling; Bulk next-generation sequencing analysis for 16S rRNA gene.	Hypothesis 1: Subsurface decomposition of *S. scrofa domesticus* tissue as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.	Changes in microbial structure were observed with the ecological indices of richness, Shannon-Wiener and Simpson diversity to investigate the decomposition impacts on soil microbiome dynamics relative to burial depth. Hypothesis accepted with bulk NGS profiling where soil microbiome composition differentiated between control and *S. scrofa domesticus* soils.	1.1 What impacts do sealed microcosms have in comparison to open and/or on site treatments? 1.2 What are the implications of bulk versus depth-specific soil microbiome profiling for accurate PMI/PBI determinations?
Study II_Chapter 4: 4 g pig skeletal muscle; 80 g Soil II (21% clay, 21% silt, 58% sandy); *Agrostis/Festuca*	Hypothesis 1: Subsurface decomposition of *S. scrofa domesticus* tissue as a mammalian	The ecological indices of richness, Shannon-Wiener and Simpson diversity showed changes in	2.1 What are the impacts of using whole cadavers/mammalian

spp plant litter; Destructively sampled treatment; Temporal 16S and 18S DGGE profiling; Partial temporal next-generation sequencing analysis for 16S rRNA gene.	proxy will change the structure and composition of the surrounding soil microbiome.	microbial structure. This was supported with NGS where compositional changes with increased abundances of Staphylococcaceae, Micrococcaceae, Alcaligenaceae for the for the *S. scrofa domesticus* tissue were observed when compared to the soil only control.	proxies compared to parts of different tissues? 2.2 What are the effects of different plant litter decompositions within the same soil when compared to human cadaver/mammalian surrogate?
	Hypothesis 2: Subsurface decomposition of *S. scrofa domesticus* tissue will effect different shifts in the surrounding soil microbiome structure and composition when compared to plant (*Agrostis/Festuca* spp) litter.	The use of ecological indices of richness, Shannon-Wiener and Simpson diversity profiling of the bacterial 16S rRNA gene seemed to be the most suitable for *S. scrofa domesticus* PMI estimation while ecological indices of the fungal 18S rRNA gene were better indicators of plant litter decomposition. NGS at genus levels of taxonomic resolution identified	2.3 What are the implications for different study start dates to correctly establish and differentiate between temporal, seasonal and annual effects?

		Sphingobacterium and *Rhodanobacter* as 16S-based taxa divergence indicators between pig and vegetation decomposition.	
	Hypothesis 3: Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and plant (*Agrostis/Festuca* spp) litter decompositions.	The use of ecological indices of richness, Shannon-Wiener and Simpson diversity revealed changes in the microbial community. NGS-based increases in abundances of *Sphingobacterium* and *Pedobacter* identified them as seasonal microbial clock indicators.	
Study III_Chapter 5: Whole piglets (1.32 – 1.8 kg); 30 – 50.4 kg Soil III (26% clay, 21% silt, 53% sandy); *Agrostis/Festuca* spp plant litter; Temporal 16S and 18S DGGE profiling; Temporal NGS analysis of 16S rRNA gene.	Hypothesis 1: Subsurface decomposition of whole *Sus scrofa domesticus* as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.	The ecological indices of richness, Shannon-Wiener and Simpson diversity revealed changes in the microbial community. Increases in abundances of Microbacteriaceae, Alcaligenaceae	a. As for Question 1.1. b. As for Question 2.2. c. As for Question 2.3. d. What impacts does cadaver/surrogate storage,

		and Sphingobacteriaceae resulted for the *S. scrofa domesticus* decomposition.	particularly freezing, have on the indigenous cadaver epinecrotic community, decomposition process and gravesoil microbiome?
	Hypothesis 2: Subsurface decomposition of whole *S. scrofa domesticus* will effect different shifts in the surrounding soil microbiome structure and composition when compared to plant (*Agrostis/Festuca* spp) litter.	The use of ecological indices revealed differences in community structure with profiling of the 16S rRNA bacterial gene more suitable for *S. scrofa domesticus* PMI estimation, while ecological indices of the fungal 18S rRNA gene were better indicators of plant litter decomposition. Likewise, increase in taxa abundances of Microbacteriaceae, Alcaligenaceae and Sphingobacteriaceae were observed for the *Sus scrofa domesticus* decomposition when compared to increases in abundances of Planctomycetaceae and	e. What effects does the age of the study animal model have on the microbiome dynamics and PMI estimation? f. What role does the cadaver/carcass size play in accurate PMI estimations?

		Comamonadaceae for the plant litter decomposition.	
	Hypothesis 3: Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and plant (*Agrostis/Festuca* spp) litter decompositions.	Hypothesis accepted as evidenced with increases in numerical abundances of several taxa such as Microbacteriaceae, Flavobacteriaceae, Alcaligenaceae and Xanthomonadaceae identified as potential seasonal indicators for summer.	
Study IV_Chapter 6: Whole piglets (1.32 – 1.8 kg); *in situ* burials in Soil IV (22% clay, 32% silt, 46% sand); *Quercus robur* leaf litter; Temporal 16S and 18S DGGE profiling; Temporal NGS analysis of 16S rRNA gene.	Hypothesis 1: *In situ* subsurface decomposition of whole *Sus scrofa domesticus* as a mammalian proxy will change the structure and composition of the surrounding soil microbiome.	Hypothesis accepted at family-level where increased relative abundances of Methylophilaceae and Flavobacteriaceae were recorded for the *S. scrofa domesticus* while increases in the relative abundances of Chitinophagaceae, Xanthomonadaceae, Hydrogenophilaceae and	4.1 As for Question 1.1. 4.2 As for Question 2.2. 4.3 As for Question 2.3. 4.4 As for Question 3.4. 4.5 As for Question 3.5. 4.6 As for Question 3.6.

		Methylococcaceae were observed for the control.	4.7 What are the *in situ* impacts of different human taphonomic proxies on the same site?
	Hypothesis 2: *In situ* subsurface decomposition of whole *S. scrofa domesticus* will effect different shifts in the surrounding soil microbiome structure and composition when compared to plant (*Quercus robur*) litter.	Increased abundances of Anaerolineaceae, Ignavibacteriaceae and Hydrogenophilaceae between the control and leaf litter while Methylophilaceae and Flavobacteriaceae were observed for the piglet treatment.	
	Hypothesis 3: Seasonal variations will influence shifts in soil microbiome structure and composition during *S. scrofa domesticus* and plant litter (*Quercus robur*) decompositions.	Increases in numerical abundances of several taxa such as Sphingomonadaceae Hydrogenophilaceae and Xanthomonadaceae identified as potential seasonal indicators for summer and autumn.	

Ideally, temporal NGS analyses of individual replicates for both the 16S bacterial and 18S fungal genes should have been made for all four studies. This would have ensured a more comprehensive understanding of the soil microbiomes and, therefore, expanded further on knowledge generated from similar studies, globally, which focus typically on the 16S bacterial gene. Nonetheless, NGS analysis in Study I of pooled DNA of samples from top, middle and bottom layers of the control and experimental soils, respectively, achieved bulk analysis of the bacterial 16S and fungal 18S rRNA gene profiles to compare, initially, the impacts of *S. scrofa domesticus* decomposition on total gravesoil microbial communities to that in a non-burial control. For Study II, triplicate DNA samples from unique time-points, which were selected on the basis of DGGE-derived ecological indices data, were pooled to start to identify: (i) temporal shifts in microbiome structure and composition; and (ii) potential postmortem/postburial and microbial clock indicators for pig and plant litter decompositions. Notwithstanding their apparent/recognised mathematical and statistical limitations, key findings from both studies suggested the potential applicability of bulk soils and/or pooled DNA samples in crime scenes where replication would, possibly, be limited or unlikely.

The importance of soil physicochemical analysis as exemplified by Dalva *et al*. (2017), where soil air pore concentrations of methane, carbon dioxide and nitrous oxide were measured in an experimental pig mass grave, is acknowledged fully. Along with commending use of nitrous oxide to locate clandestine graves, the workers revealed seasonal variations in CO_2 concentrations with the highest values recorded during summer periods. Therefore, such tools can be complemented with forensic ecogenomic analyses to provide detailed microbial signature profiling during the decomposition timelines.

Although DGGE and NGS are, respectively, the most used and high throughput molecular techniques for characterising complex microbial communities, they have their inherent advantages and disadvantages [1.9.2]. Also, both methods require DNA extraction and PCR

and are, therefore, subject to the attendant biases of these steps. These must be recognised and accounted for during subsequent data presentation and application specifically in forensic contexts. Another important factor with these techniques is the use and design of PCR primers. Furthermore, whilst the primer sequences used in this research programme were reported previously in the literature, primer design and use can target specific functional groups such as ammonia oxidising, proteolytic, lipolytic and carbolytic communities to: (i) avoid over-generalisation of the microbial communities; and (ii) complement established analyses [1.9; Table 1.1].

The importance and value of replicated studies are recognised widely in scientific research including in several decomposition studies that were designed to illustrate the applicability of microbial ecology techniques in forensic contexts. According to Moreau *et al*. (2015) and Schoenly *et al*. (2015), for example, unreplicated studies, or pseudoreplications, can limit the precision and mathematical/statistical/application significances of the generated data. Also, the relevance of study replication, method validation and application of robust statistical analyses in novel forensic contexts was exemplified by Pasternak *et al*. (2012) who investigated two commercial soil DNA extraction kits, two widely-used microbial community profiling techniques and the efficacy of different restriction enzymes for T-RFLP. Therefore, whilst their limitations are recognised fully, Study I still provided a potential direct comparison between a non-burial and clandestine grave scenario relative to depth and Study III was the first where whole piglet decomposition was studied in the subsurface with and without plant litter. For Study I and III, one-way ANOVA was applied to assess statistical significant differences between the control and treatment soils. These were analysed further with principal component analysis, NMDS, PERMANOVA and pair-wise multiple comparisons after a multi-way ANOVA with Tukey (HSD) *post hoc* test for robust data interpretation. For Studies II and IV with triplicates, data were analysed with two-way repeat measure ANOVA and subsequent Tukey (HSD) *post hoc* test. Further data analyses such as NMDS, principal component analysis, PERMANOVA, ANOVA and pair-

wise multiple comparisons after a multi-way ANOVA with Tukey (HSD) *post hoc* test were applied for additional scrutiny to determine whether measurable significant differences resulted with treatment/carbon source, time and season.

Although the four experimental designs may not be ideal and prevent direct cross-study comparisons, they afforded the opportunity to assess the microbial communities of different soil types and the impacts of several carbon sources decompositions relative to depth, time and season. Therefore, the strengths and limitations of the current research programme must inform future work relative to experimental designs, whole or components of *S. scrofa domesticus*, plant litter, complementation of appropriate physicochemical and molecular techniques and mathematical/statistical analyses.

7.4 Future work

Against the new knowledge developed and study-specific research questions identified above, additional key paucities remain. These include: to what extent do abiotic factors affect the microbial community; what are the consistent effects of seasonal differences on the microbial community; and how should the impacts of global climate change be investigated with regards to PMI estimation. Therefore, future decomposition investigations should include comprehensive study designs and analyses and consider: various animal models of different sizes and ages relative to nutrient load and composition; study animals of similar age and size in different *in situ* soils and local climates; effects of cadaver/surrogate storage (freezing); parallel profiling of the cadaver/taphonomic proxy microbiome and gravesoil microbiota extending to both archaeal and fungal communities; soil physicochemical analyses; gaseous and volatile organic carbon emissions; and volatile fatty acid profiling relative to soil pH. All of these must be implemented parallel to both affordable microbial community profiling techniques and more high-throughput platforms such as NGS.

The characterisation of soil microbial communities by culture-independent techniques has indicated their potential forensic applicability in estimating postmortem interval (PMI),

nevertheless accurate PMI estimation still poses a challenge since soil is a complex heterogeneous habitat with diverse microbial communities. The current research programme revealed further the complexity in soil microbial communities along seasonal timelines relative to the carbon sources. Subject to further research into these aspects and relevant validation, forensic ecogenomics can be incorporated into existing tools such as entomology, cadaver dogs, soil physicochemical analyses, geophysical analysis to resolve cases of transit graves (where a body has been removed), clandestine graves and complete decomposition (juvenile cases). It may further help forensic practitioners in understanding and interpreting crime scenes with and without decomposing cadavers.

Also, future subsurface investigations should, as much as is possible, entail analysis of the decomposition stages by monitoring physical changes of (Adlam and Simmons 2007), and total body score (Moffatt *et al*., 2016) determinations for, decomposing mammalian taphonomic proxies. Furthermore, as illustrated by some emerging but aboveground studies (Pechal *et al*., 2013), innovative experimental designs are required to elucidate microbiome-insect interactions (*re* chemical signals) in belowground decomposition scenarios, including in the presence of plant litter (Teuben and Roelofsma 1990). These complementary approaches at the interface of forensic ecogenomics, forensic entomology and forensic chemistry should provide additional metadata for enhanced subsurface postmortem interval and time-since-burial estimations.

Notwithstanding the recorded divergences where some families and genera could be useful microbial indicators, the effects of time and season must be explored comprehensively and separately. They mandate further robust and protracted experimental designs that consider: (i) different start (D0) dates, possibly from different seasons during the same calendar year; and (ii) the same start and end dates during similar seasons but in consecutive/different years. Thus the identification/differentiation of core (non-transient) temporal and seasonal microbial clocks for the same soil type and animal model would be attained.

Initiatives such as the human microbiome (Turnbaugh *et al*., 2007) and thanatomicrobiome (Javan *et al*., 2016) projects aimed to identify and differentiate core and transient members that can be used to establish health and disease states, and be adopted as microbial clock indicators for enhanced PMI determinations, respectively. In parallel, and although outwith the forensic context, multi-team studies *e.g*. Stone *et al*., (2016) have illustrated the utility of analysing microbial ecological dynamics of soils under similar and different management regimen, between similar soil types, and/or across geographical locations with different climatic conditions. Similarly, the effects of soil depth (surface, subsurface) and season (summer, winter) on microbial community size, structure and function were exemplified by Blume *et al*. (2002) who recorded sesonal effects on subsurface population structure and activity. Notwithstanding this, changes and similarities in soil ecology are typically dependent on several factors including but not limted to experimental design, analytical method(s), site, soil type, depth, vegetation cover. Therefore, concerted efforts are required for soil microbiome profiling both aboveground and in the subsurface to establish the applicability of temporal- and seasonal-focused ecogenomic analyses in forensic scenarios. Ultimately, the importance of site-specific non-burial controls in protracted decomposition-based forensic microbiology investigations, to elucidate the occurrences of both unique and universal microbial taxa, cannot be overemphasised.

Although not included in Chapter 6, geophysical analysis was conducted for the *in situ* study. Overall, the geomagnetic techniques proved inappropriate due to previous use of the site as a landfill. In particular, it was impossible to calibrate the instrument sensors on or near the site so this was done off-site. Earth electrical resistance survey can be an effective method for detecting cut features and its efficacy in locating covered and uncovered cadavers was illustrated in field tests by Pringle *et al*. (2012a). For the *in situ* study, the resistance data were relatively consistent over the 10-months period. Temporal and spatial variations, with regions of high and low resistance, were detected across broad sections of the site with very small differences recorded for the various pits, with and without buried material. Some

additional slight variations were detected and were most likely due to the prevailing weather and ground conditions preceding and during each survey. Generally, pit size made electrical resistance detection difficult. Also, it is unlikely that any physical changes in the pits and graves, such as piglet, would have been sustained and/or sufficient to effect the recorded soil macrochanges.

The GPR was adopted since previous studies have demonstrated its efficacy in locating graves (Pringle *et al*., 2012b; Schultz and Martin, 2012), and it provided the clearest evidence for detecting the experimental pits. Time-slices/bites of both the 500 and 800 MHz antennae data from depths of 0.08 - 0.15 m showed clear reflections of all nine 'graves'. Nonetheless, they appeared to represent the outlines of the pits and metal meshes where present rather than the piglet carcasses *per se*, with little differences between the piglets, vegetation and control pits. Although the 500 MHz antenna gave positive results, the 800 MHz antenna proved more effective for locating the burials, which would be expected due to the relatively shallow depths used. Therefore, this and similar standard crime scene investigative tools, should be used in future studies to show how forensic ecogenomics fits into and has the potential to augment existing forensic investigations.

Finally, while the studies in the current research programme were made with pigs as surrogates for human cadavers, the use of human cadavers would provide more robust and conclusive data sets for forensic practitioners in understanding the complex relationships between cadaver and soil microbial community dynamics.

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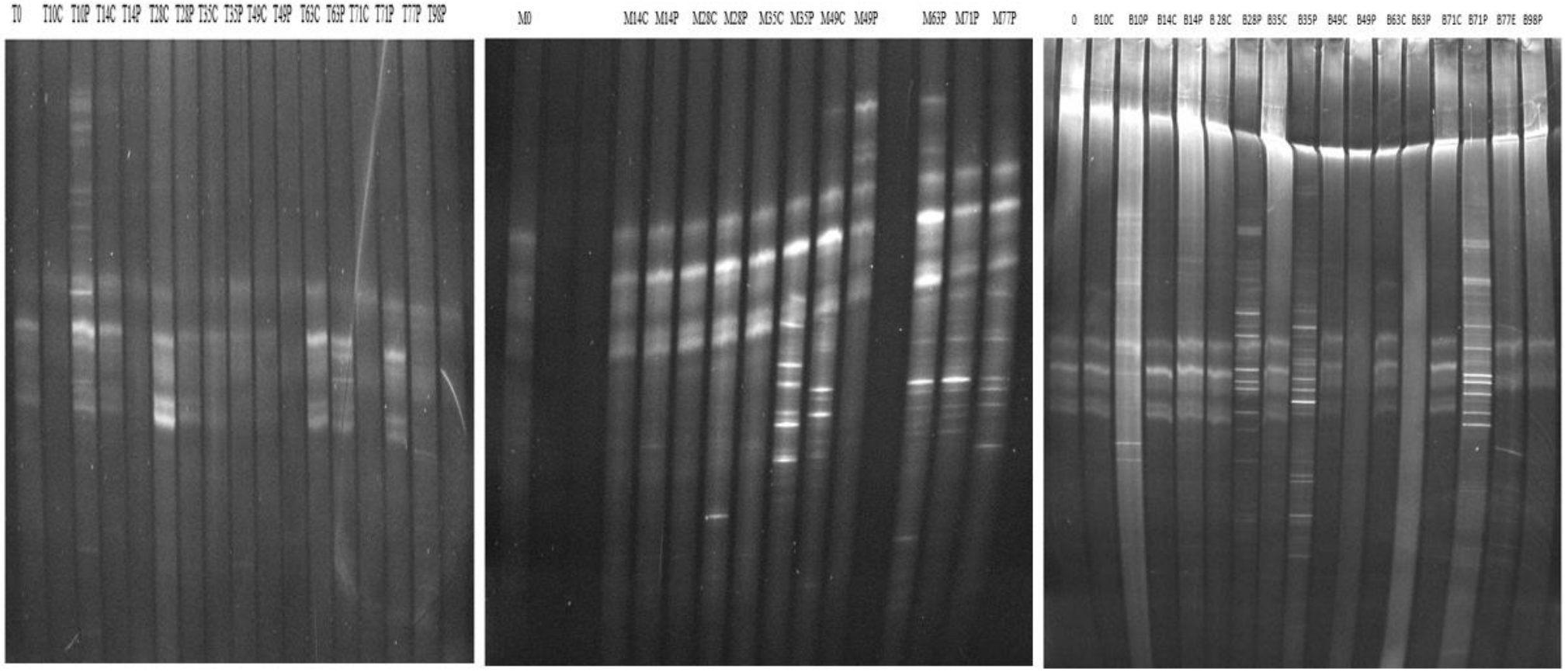
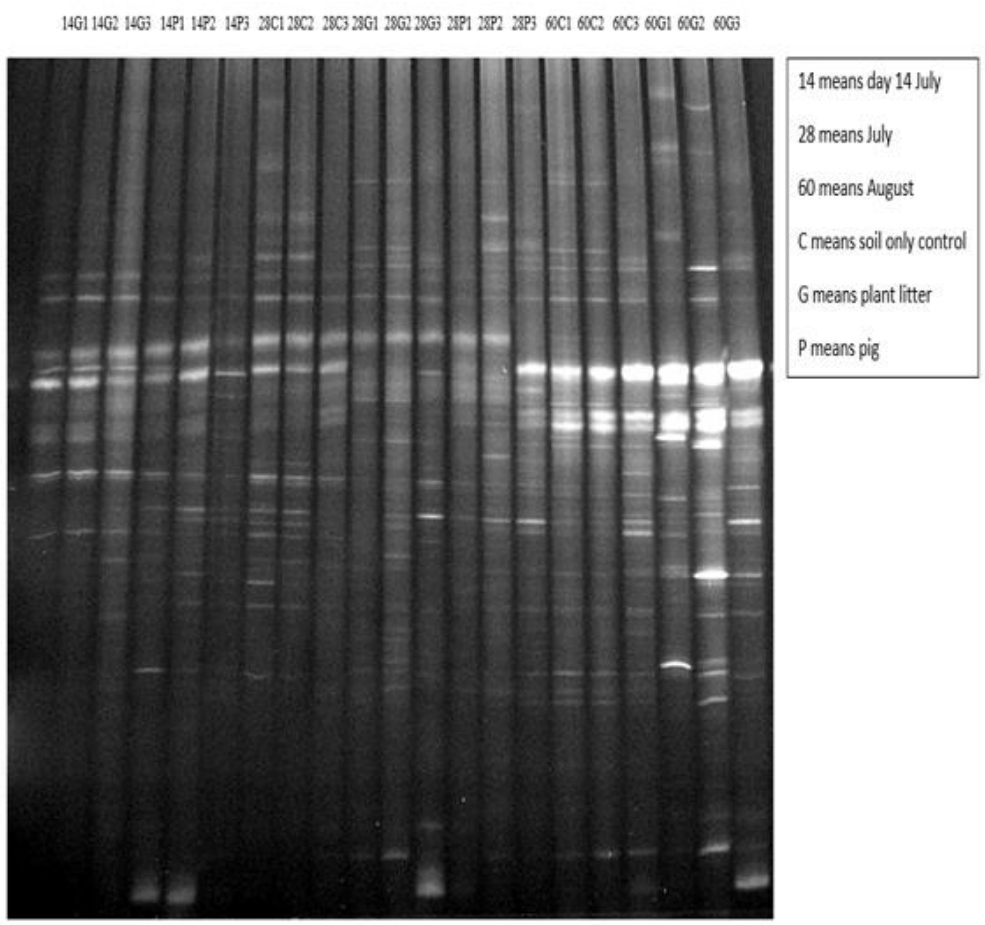
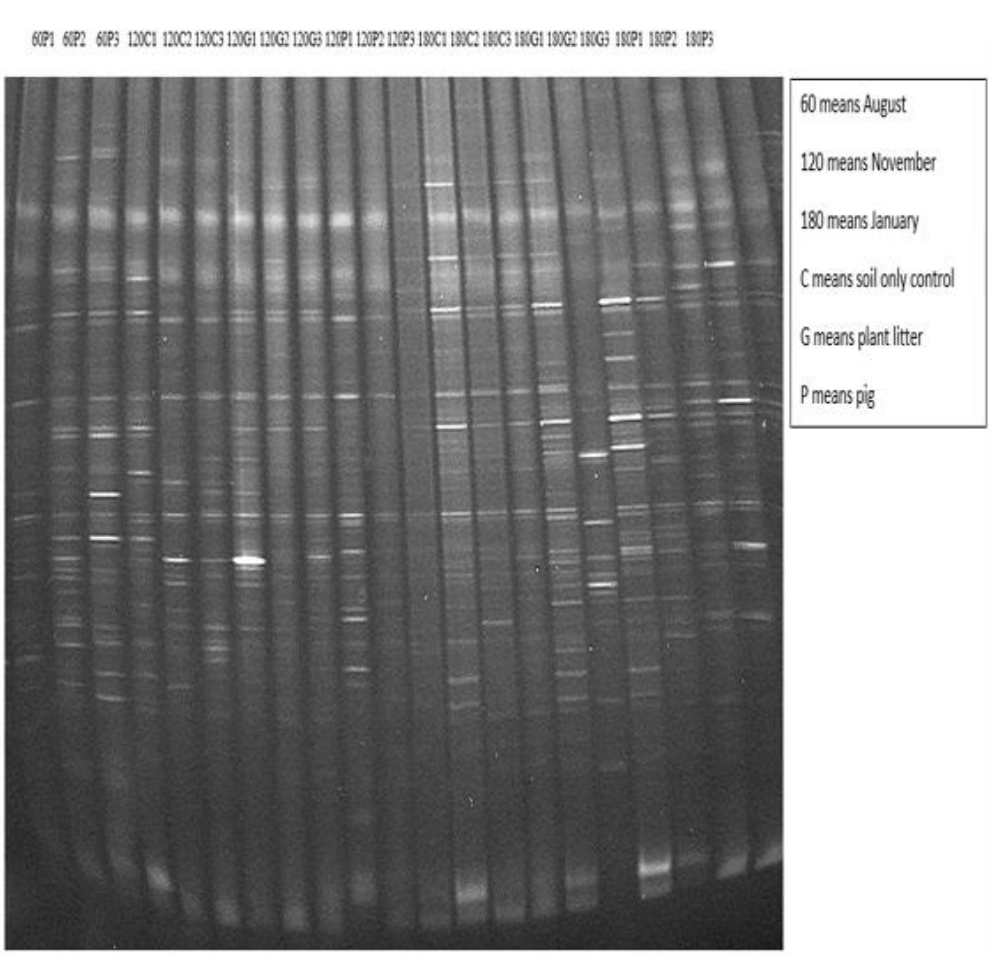
Appendix A: Study I 16S DGGE gel for 98 days decomposition study (July – September 2011).

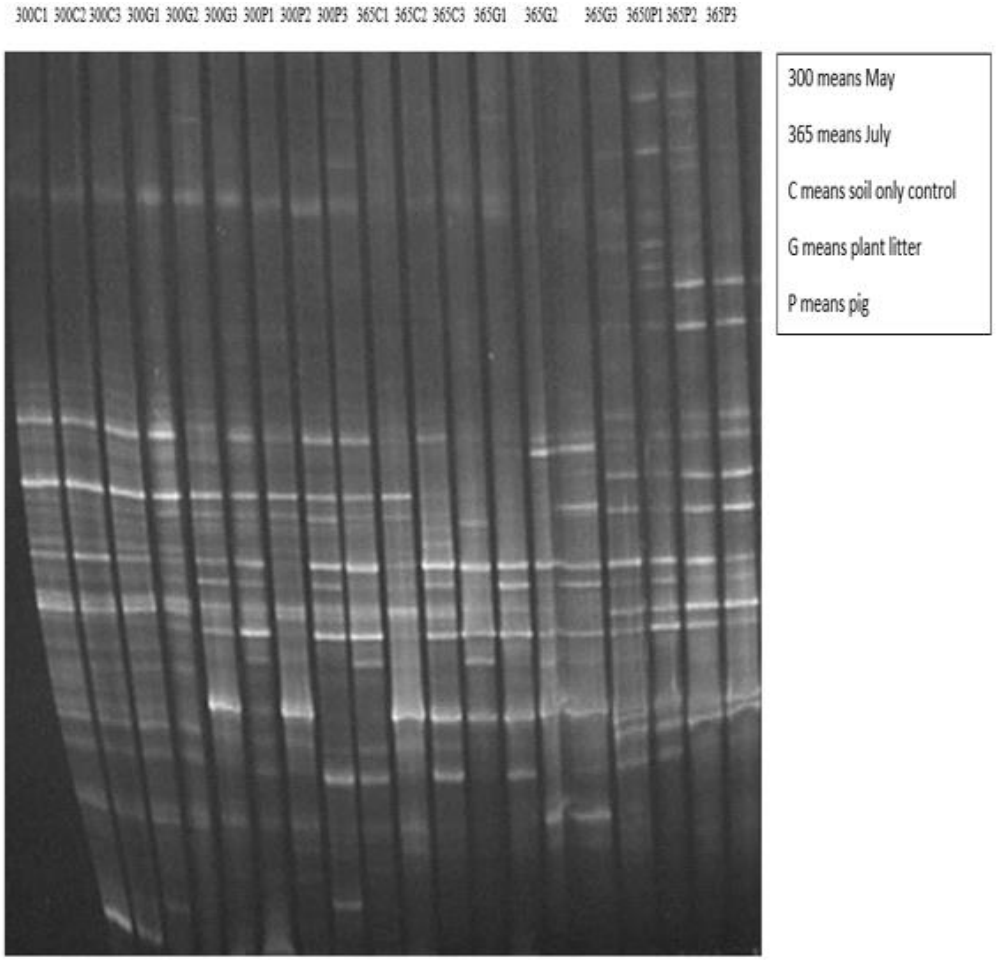


Where T means Top, M means middle, B means bottom section, C means control, P means pig and 0 – 98 means days of sample collection.

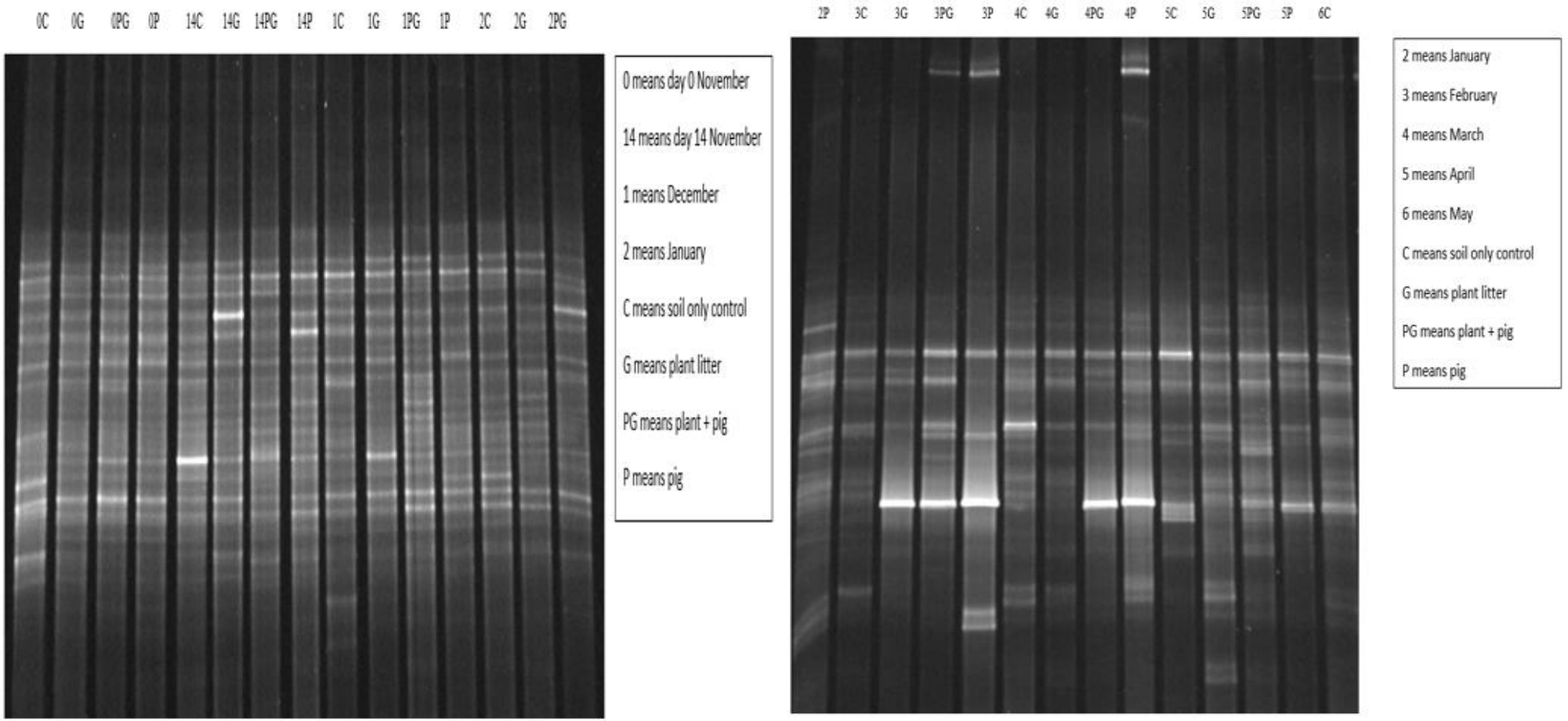
Appendix B: Study II 16S DGGE gel from day 0 to day 365 (July 2013 – July 2014).

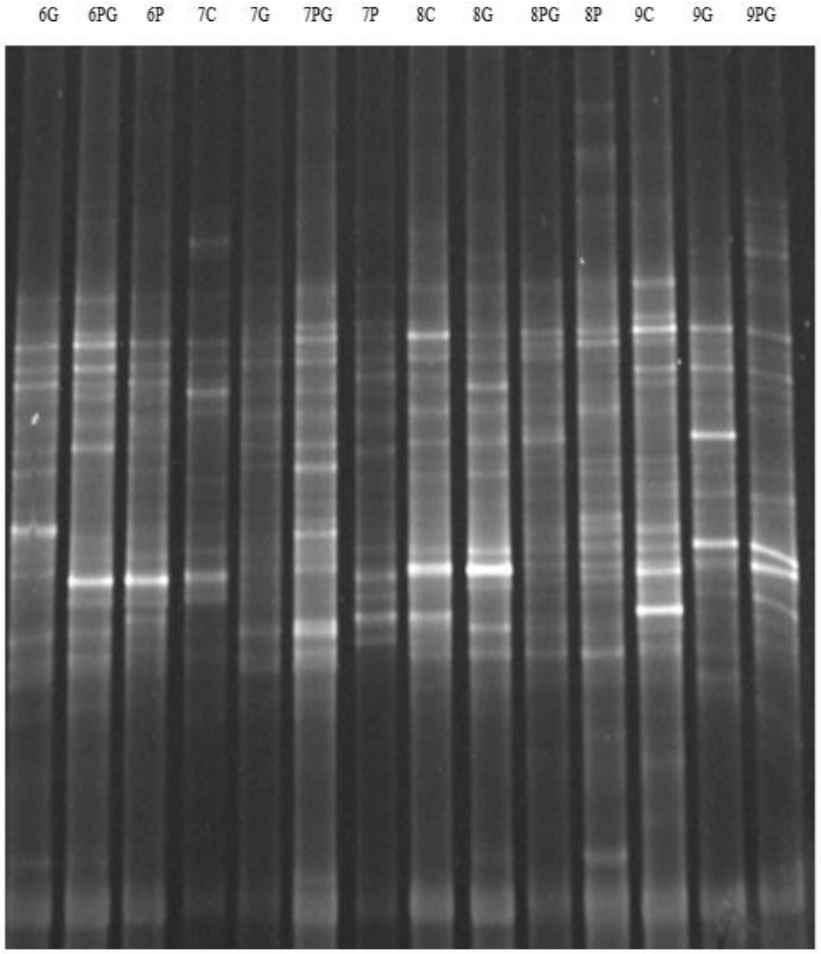



6OP1 6OP2 6OP3 120C1 120C2 120C3 120G1 120G2 120G3 120P1 120P2 120P3 180C1 180C2 180C3 180G1 180G2 180G3 180P1 180P2 180P3
60 means August
120 means November
180 means January
C means soil only control
G means plant litter
P means pig


300C1 300C2 300C3 300G1 300G2 300G3 300P1 300P2 300P3 365C1 365C2 365C3 365G1 365G2 365G3 365OP1 365P2 365P3
300 means May
365 means July
C means soil only control
G means plant litter
P means pig

Appendix C: Study III 16S DGGE gel from November 2014 to September 2015.





6 means May

7 means June

8 means July

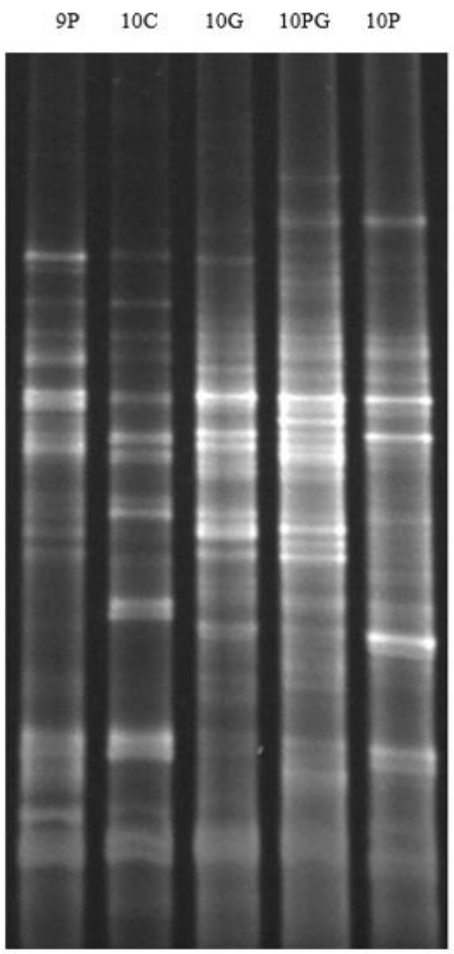
9 means August

C means soil only control

G means plant litter

PG means plant + pig

P means pig



9 means August

10 means September

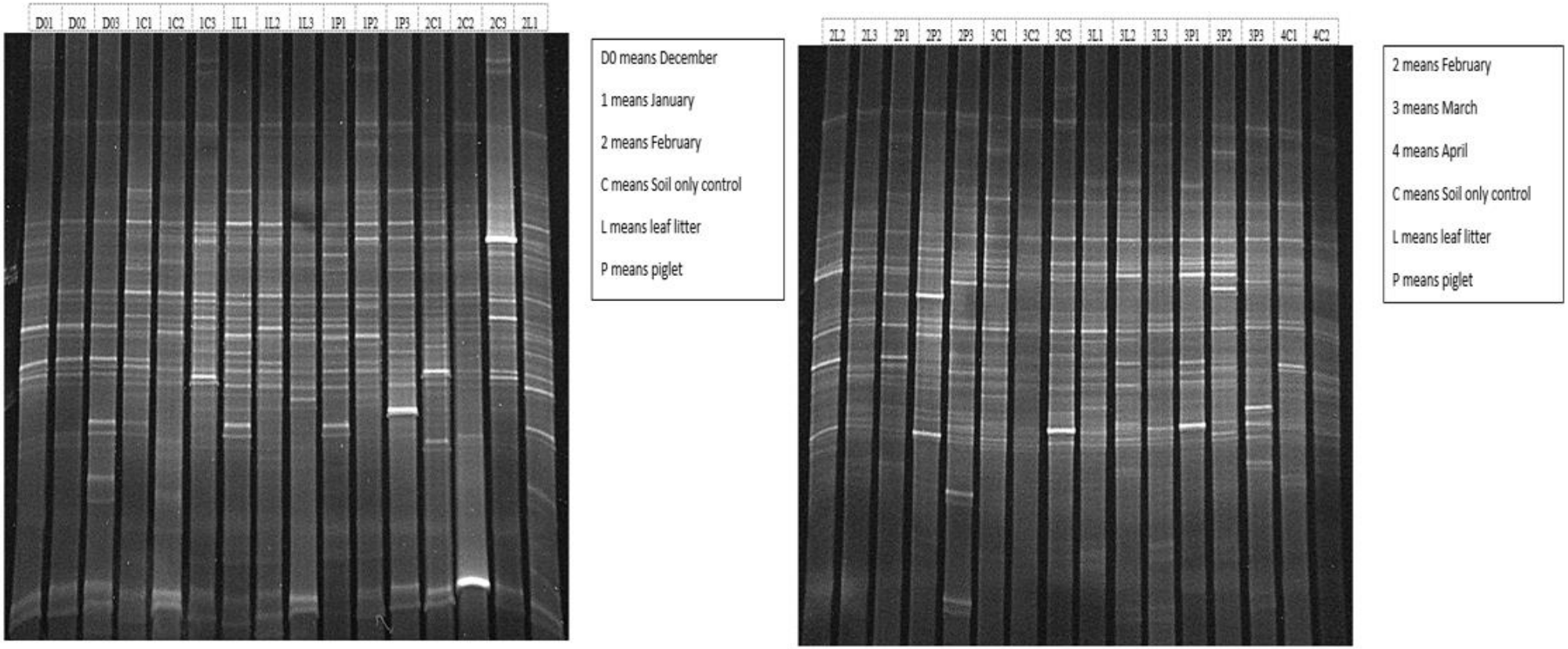
C means soil only control

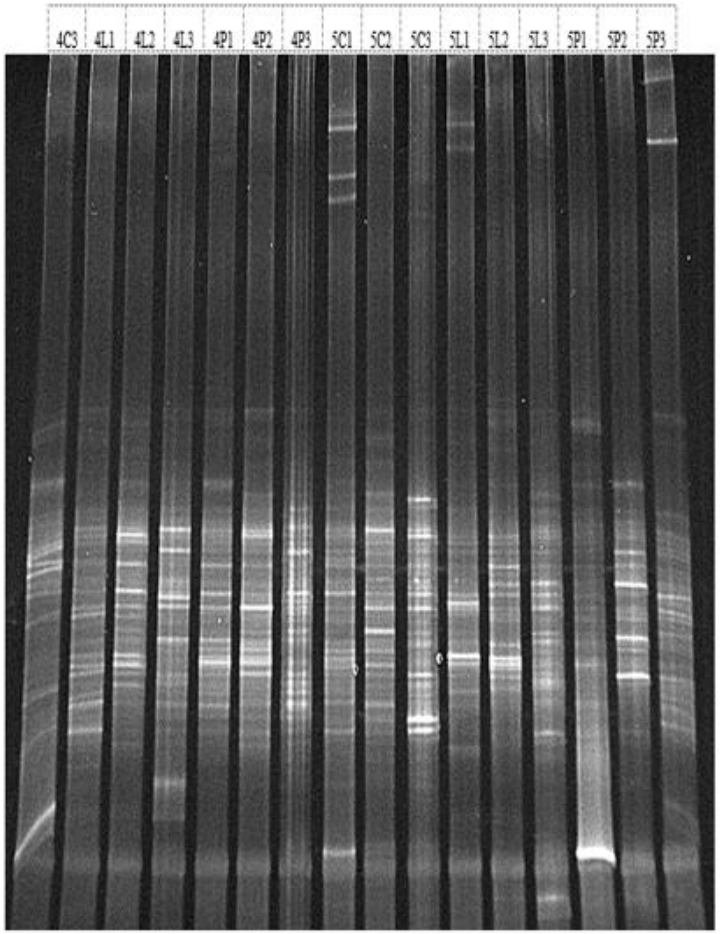
G means plant litter

PG means plant + pig

P means pig

Appendix D: Study IV 16S DGGE gel from December 2014 to September 2015.





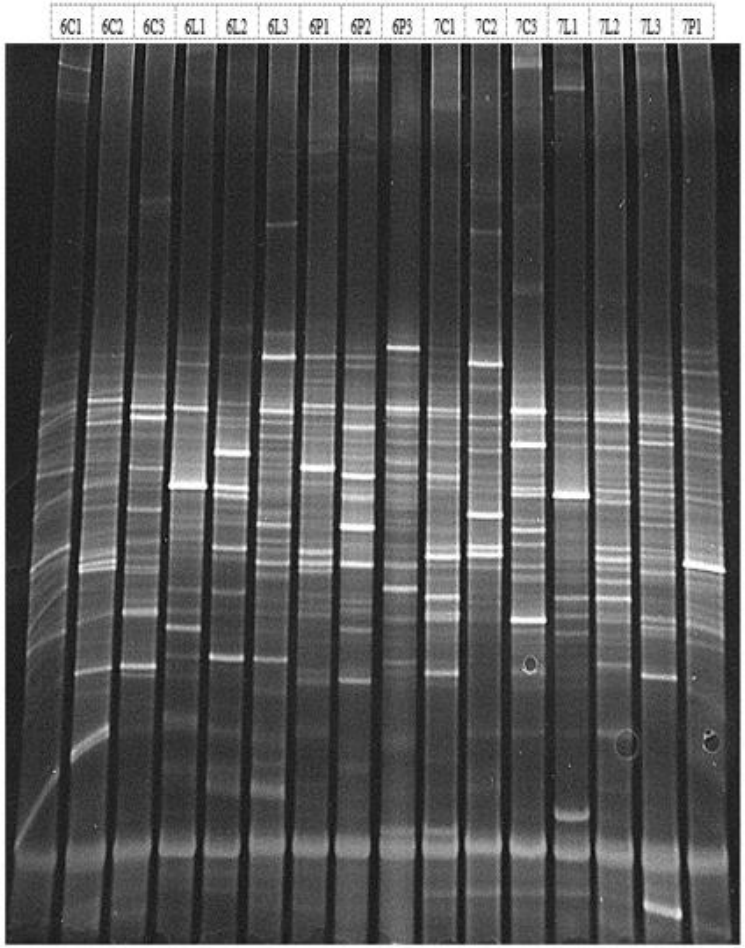
4 means April

5 means May

C means Soil only control

L means leaf litter

P means piglet



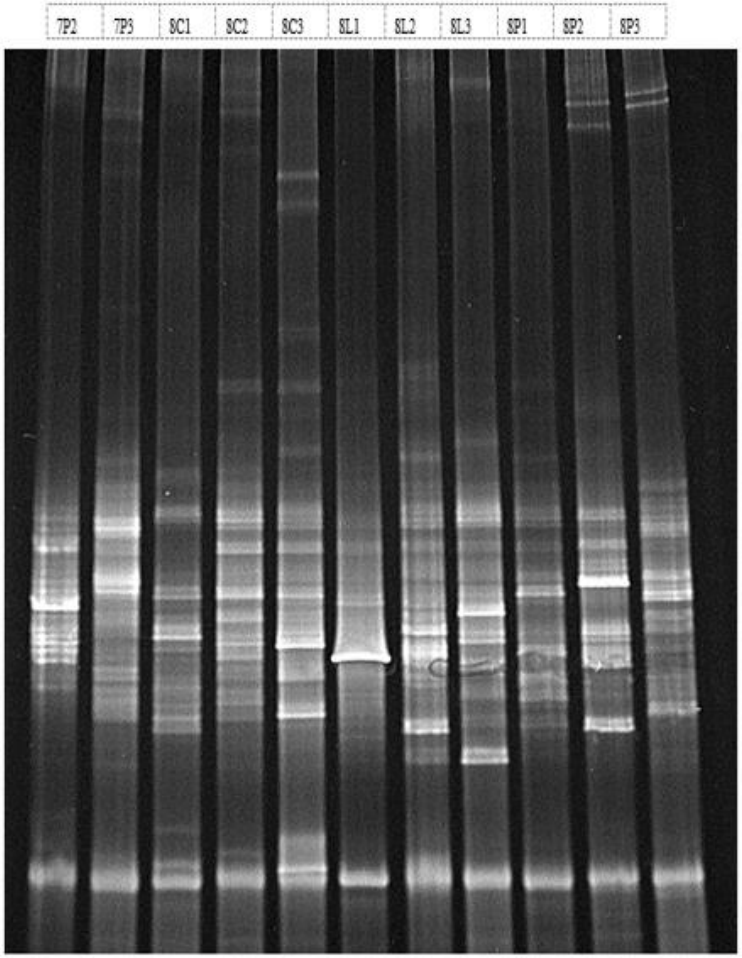
6 means June

7 means July

C means Soil only control

L means leaf litter

P means piglet



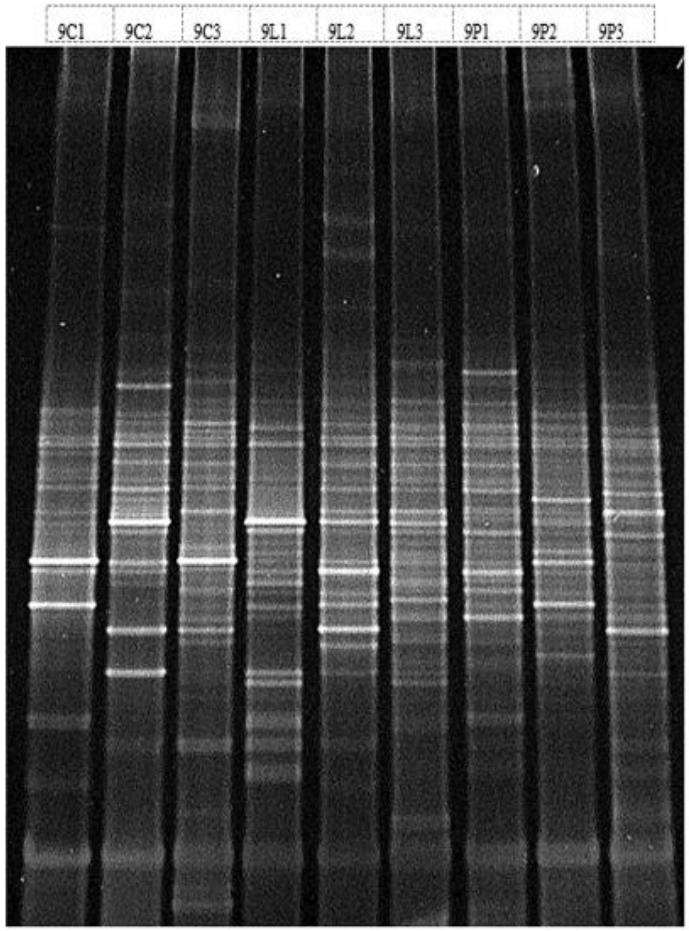
7 means July

8 means August

C means Soil only control

L means leaf litter

P means piglet



9 means September

C means Soil only control

L means leaf litter

P means piglet